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(54) Title: PROCESSES FOR ENHANCED PRODUCTION OF PANTOTHENATE

(57) Abstract: The present invention features improved methods for producing pantoate and pantothenate utilizing microorganisms having modified pantothenate biosynthetic enzyme activities. In particular, the invention features methods for reducing byproduct formation and increasing yields and purity of desired product. Recombinant microorganisms and conditions for culturing same are also are featured. Also featured are compositions produced by such microorganisms.

PROCESSES FOR ENHANCED PRODUCTION OF PANTOTHENATE

Related Applications

The present invetion claims the benefit of prior-filed provisional Patent Application Serial No. 60/262,995, filed January 19, 2001 (pending). The present invention is also related to U.S. Patent Application Serial No. 09/667,569, filed September 21, 2000 (pending), which is a continuation-in-part of U.S. Patent Application Serial No. 09/400,494, filed September 21, 1999 (abandoned). U.S. Patent Application Serial No. 09/667,569 also claims the benefit of prior-filed provisional Patent Application Serial No. 60/210,072, filed June 7, 2000, provisional Patent Application Serial No. 60/221,836, filed July 28, 2000, and provisional Patent Application Serial No. 60/227,860, filed August 24, 2000. The entire content of each of the above-referenced applications is incorporated herein by this reference.

Background of the Invention

Pantothenate, also known as pantothenic acid or vitamin B5, is a member of the B complex of vitamins and is a nutritional requirement for mammals, including livestock and humans (*e.g.*, from food sources, as a water soluble vitamin supplement or as a feed additive). In cells, pantothenate is used primarily for the biosynthesis of coenzyme A (CoA) and acyl carrier protein (ACP). These coenzymes function in the metabolism of acyl moieties which form thioesters with the sulfhydryl group of the 4'-phosphopantetheine portion of these molecules. These coenzymes are essential in all cells, participating in over 100 different intermediary reactions in cellular metabolism.

The conventional means of synthesizing pantothenate (in particular, the bioactive D isomer) is *via* chemical synthesis from bulk chemicals, a process which is hampered by excessive substrate cost as well as the requirement for optical resolution of racemic intermediates. Accordingly, researchers have recently looked to bacterial or microbial systems that produce enzymes useful in pantothenate biosynthesis processes (as bacteria are themselves capable of synthesizing pantothenate). In particular, bioconversion processes have been evaluated as a means of favoring production of preferred isomer of pantothenic acid. Moreover, methods of direct microbial synthesis have recently been examined as a means of facilitating D-pantothenate production.

There is still, however, significant need for improved pantothenate production processes, in particular, for microbial processes optimized to produce higher yields of desired product.

Summary of the Invention

The present invention relates to improved processes (*e.g.*, microbial syntheses) for the production of pantothenate. In particular, the present inventors have discovered that deregulation of the pantothenate biosynthetic pathway and/or deregulation of the isoleucine-valine (*ilv*) pathway in microorganisms, in addition to producing significantly increased pantoate and/or pantothenate titers, results in the synthesis of an alternate product, namely [R]-3-(2-hydroxy-3-methyl-butyrylamino)-propionic acid or 3-(2-hydroxy-3-methyl-butyrylamino)-propionic acid ("HMBPA"), referred to interchangeably herein as "β-alanine 2-(*R*)-hydroxyisolvalerate", "β-alanine 2-hydroxyisolvalerate", "β-alanyl-α-hydroxyisovalerate" and/or "fantothenate". The pathway leading to HMBPA (referred to herein as the "HMBPA biosynthetic pathway") involves certain enzymes conventionally associated with pantothenate and/or isoleucine-valine (*ilv*) biosynthesis, which when overexpressed, are capable of additionally participating in the HMBPA biosynthetic pathway. In particular, the pathway includes conversion of α-ketoisovalerate to [R]-2-hydroxyisovalarate (α-HIV), catalyzed by a reductase activity (*e.g.*, PanE1, PanE2 and/or IlvC activities), followed by condensation of α-HIV with β-alanine, catalyzed by PanC activity. As the alternative HMBPA biosynthetic pathway competes for key precursors of pantothenate biosynthesis, namely α-ketoisovalerate (α-KIV) and β-alanine, and also competes for enzymes conventionally associated with pantothenate biosynthesis, it is desirable to decrease or eliminate HMBPA biosynthesis in order to effectively increase pantothenate biosynthesis.

Accordingly, in one aspect the present invention features a process for the production of a HMBPA-free pantothenate composition that includes culturing a microorganism having a deregulated pantothenate biosynthetic pathway under conditions such that a HMBPA-free pantothenate composition is produced. In another aspect, the invention features a process for the production of a HMBPA-free pantothenate composition that involves culturing a microorganism having a deregualted pantothenate biosynthetic pathway and a deregulated isoleucine-valine (*ilv*) biosynthetic pathway, said microorganism having PanB activity regulated such that a HMBPA-free pantothenate composition is produced. Yet another aspect of the invention features a process for the production of a HMBPA-free pantothenate composition that involves culturing a microorganism having a deregualted pantothenate biosynthetic pathway and a deregulated isoleucine-valine (*ilv*) biosynthetic pathway, the microorganism having PanE activity regulated such that a HMBPA-free pantothenate composition is produced. In yet another aspect, the invention features a process for the production of a HMBPA-free pantothenate composition that involves culturing a microorganism having a

deregualted pantothenate biosynthetic pathway and a deregulated isoleucine-valine (*ilv*) biosynthetic pathway, the microorganism having IlvC activity regulated such that a HMBPA-free pantothenate composition is produced. In yet another aspect, the invention features a process for the production of a HMBPA-free pantothenate composition that involves culturing a microorganism having a deregualted pantothenate biosynthetic pathway and a deregulated isoleucine-valine (*ilv*) biosynthetic pathway, said microorganism having PanB and PanE activites regulated such that a HMBPA-free pantothenate composition is produced. In yet another aspect, the invention features a process for the production of a HMBPA-free pantothenate composition that involves culturing a microorganism having a deregualted pantothenate biosynthetic pathway and a deregulated isoleucine-valine (*ilv*) biosynthetic pathway, said microorganism having PanB and IlvC activites regulated such that a HMBPA-free pantothenate composition is produced. Compositions produced according to the above-described methodologies are also featured as are microorganisms utilized in said methodologies. Also featured are processes for the production of a selectively mixed pantothenate:HMBPA compositions.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

Brief Description of the Drawings

Figure 1 is a schematic representation of the pantothenate and isoleucine-valine (*ilv*) biosynthetic pathways. Pantothenate biosynthetic enzymes are depicted in bold and their corresponding genes indicated in italics. Isoleucine-valine (*ilv*) biosynthetic enzymes are depicted in bold italics and their corresponding genes indicated in italics.

Figure 2 is a schematic representation of the biosynthetic pathway leading to the formation [R]-3-(2-hydroxy-3-methyl-butyrylamino)-propionic acid ("HMBPA") in *B. subtilis*.

Figure 3 is a schematic depiction of the structure of [R]-3-(2-hydroxy-3-methyl-butyrylamino)-propionic acid ("HMBPA").

Figure 4 is a HPLC chromatogram of a sample of medium from a 14 L fermentation of PA824.

Figure 5 is a mass spectrum depicting the relative monoisotopic mass of 3-(2-hydroxy-3-methyl-butyrylamino)-propionic acid.

Figure 6 depicts an alignment of the C-terminal amino acids from known or suspected PanB proteins.

Figure 7 is a schematic representation of the construction of the plasmid pAN624.

Figure 8 is a schematic representation of the construction of the plasmid pAN620.

Figure 9 is a schematic representation of the construction of the plasmid pAN636.

Figure 10 is a schematic representation of the construction of the plasmid pAN637 which allows selection for single or multiple copies using chloramphenicol.

Detailed Description of the Invention

The present invention is based, at least in part, on the discovery of an alternative biosynthetic pathway in recombinant microorganisms which utilizes certain pantothenate and/or isoleucine-valine (*ilv*) biosynthetic enzymes and precursors to make a byproduct or side product called [R]-3-(2-hydroxy-3-methyl-butyrylamino)-propionic acid ("HMBPA"). In particular, it has been discovered that bacteria that have been engineered to have deregulated pantothenate biosynthetic and/or isoleucine-valine (*ilv*) biosynthetic pathways are capable of generating HMBPA from α-ketoisovalerate (α-KIV), a key product of the isoleucine-valine (*ilv*) biosynthetic pathway and precursor of the pantothenate biosynthetic pathway. Production of HMBPA in bacteria utilizes at least the pantothenate biosynthetic enzymes ketopantoate reductase (the *panE* gene product), the *panE2* gene product and/or acetohydroxyisomeroreductase (the *ilvC* gene product) and results from the condensation of [R]-2-hydroxyisovaleric acid (α-HIV), formed by reduction of α-KIV, and β-alanine, the latter reaction being catalyzed by the pantothenate biosynthetic enzyme pantothenate synthetase (the *panC* gene product). The substrates α-KIV and β-alanine can be utilized for both pantothenate production and HMBPA production, β-alanine being provided, for example, by feeding and/or increased aspartate-α-decarboxylate activity (the *panD* gene product).

In order to decrease or eliminate competition for pantothenate biosynthesis precursors and/or biosynthetic enzymes, it is desireable to selectively regulate certain enzymes such that production is shifted away from HMBPA and towards pantoate/pantothenate. Preferably, microorganisms having a deregulated pantothenate biosynthetic pathway and/or a deregulated isoleucine-valine (*ilv*) biosynthetic pathway are further engineered such that PanB and/or PanE are selectively

regulated. Selective regulation of PanB and/or PanE includes an optimization of levels of these enzymes such that production flows towards pantoate/pantothenate.

In particular, the invention features methods of producing compositions having increased ratios of pantothenate to HMBPA, preferably HMBPA-free pantothenate compositions. As used herein, the phrase "HMBPA-free pantothenate composition" describes a composition including pantothenate which is free of HMBPA and/or substantially free of HMBPA such that said composition includes insignificant amounts of HMBPA (*i.e.*, if HMBPA is present, it is present at a sufficiently low level or concentration relative to the level or concentration of pantothanate such that the composition can be considered HMBPA-free for technological, scientific and/or industrial purposes). Preferably, an HMBPA-free pantothenate composition includes pantothenate and, if HMBPA is present, it is present at a ratio of 10:100 (*i.e.*, 10% HMBPA versus 90% pantothenate, for example, as determined by comparing the peak areas when a sample of product is analyzed by HPLC) or less. More preferably, an HMBPA-free pantothenate composition includes pantothenate and, if HMBPA is present, it is present at a ratio of 9:100 (*i.e.*, 9% HMBPA versus 91% pantothenate) or less. Even more preferably, a HMBPA-free pantothenate composition includes pantothenate and, if HMBPA is present, it is present at a ratio of 8:100 (*i.e.*, 8% HMBPA versus 92% pantothenate) or less, 7:100 (*i.e.*, 7% HMBPA versus 93% pantothenate) or less, 6:100 (*i.e.*, 6% HMBPA versus 94% pantothenate) or less or 5:100 (*i.e.*, 5% HMBPA versus 95% pantothenate) or less. Even more preferably, a HMBPA-free pantothenate composition includes pantothenate and, if HMBPA is present, it is present at a ratio of 0.5:100 (*i.e.*, 0.5% HMBPA versus 99.5% pantothenate) or less, 0.2:100 (*i.e.*, 0.2% HMBPA versus 99.8% pantothenate) or less or 0.1:100 (*i.e.*, 0.1% HMBPA versus 99.9% pantothenate) or less. Values and ranges included and/or intermediate of the values set forth herein are also intended to be within the scope of the present invention.

In one embodiment, the invention features a process for the production of a HMBPA-free pantothenate that includes culturing a microorganism having a deregulated pantothenate biosynthetic pathway under conditions such that a HMBPA-free pantothenate composition is produced. The term "pantothenate biosynthetic pathway" includes the biosynthetic pathway involving pantothenate biosynthetic enzymes (*e.g.*, polypeptides encoded by biosynthetic enzyme-encoding genes), compounds (*e.g.*, substrates, intermediates or products), cofactors and the like utilized in the formation or synthesis of pantothenate. The term "pantothenate biosynthetic pathway" includes the biosynthetic pathway leading to the synthesis of pantothenate in

microorganisms (*e.g.*, *in vivo*) as well as the biosynthetic pathway leading to the synthesis of pantothenate *in vitro*.

As used herein, a microorganism "having a deregulated pantothenate biosynthetic pathway" includes a microorganism having at least one pantothenate biosynthetic enzyme deregulated (*e.g.*, overexpressed) (both terms as defined herein) such that pantothenate production is enhanced (*e.g.*, as compared to pantothenate production in said microorganism prior to deregulation of said biosynthetic enzyme or as compared to a wild-type microorganism). The term "pantothenate" includes the free acid form of pantothenate, also referred to as "pantothenic acid" as well as any salt thereof (*e.g.*, derived by replacing the acidic hydrogen of pantothenate or pantothenic acid with a cation, for example, calcium, sodium, potassium, ammonium), also referred to as a "pantothenate salt". The term "pantothenate" also includes alcohol derivatives of pantothenate. Preferred pantothenate salts are calcium pantothenate or sodium pantothenate. A preferred alcohol derivative is pantothenol. Pantothenate salts and/or alcohols of the present invention include salts and/or alcohols prepared *via* conventional methods from the free acids described herein. In another embodiment, a pantothenate salt is synthesized directly by a microorganism of the present invention. A pantothenate salt of the present invention can likewise be converted to a free acid form of pantothenate or pantothenic acid by conventional methodology. Preferably, a microorganism "having a deregulated pantothenate biosynthetic pathway" includes a microorganism having at least one pantothenate biosynthetic enzyme deregulated (*e.g.*, overexpressed) such that pantothenate production is 1 g/L or greater. More preferably, a microorganism "having a deregulated pantothenate biosynthetic pathway" includes a microorganism having at least one pantothenate biosynthetic enzyme deregulated (*e.g.*, overexpressed) such that pantothenate production is 2 g/L or greater.

The term "pantothenate biosynthetic enzyme" includes any enzyme utilized in the formation of a compound (*e.g.*, intermediate or product) of the pantothenate biosynthetic pathway. For example, synthesis of pantoate from α-ketoisovalerate (α-KIV) proceeds *via* the intermediate, ketopantoate. Formation of ketopantoate is catalyzed by the pantothenate biosynthetic enzyme PanB or ketopantoate hydroxymethyltransferase (the *panB* gene product). Formation of pantoate is catalyzed by the pantothenate biosynthetic enzyme PanE1 or ketopantoate reductase (the *panE1* gene product). Synthesis of β-alanine from aspartate is catalyzed by the pantothenate biosynthetic enzyme PanD or aspartate-α-decarboxylase (the *panD* gene product). Formation of pantothenate from pantoate and β-alanine (*e.g.*, condensation) is catalyzed by the pantothenate biosynthetic enzyme PanC or pantothenate synthetase (the *panC*

gene product). Pantothenate biosynthetic enzymes may also perform an alternative function as enzymes in the HMBPA biosynthetic pathway described herein.

Accordingly, in one embodient, the invention features a process for the production of a HMBPA-free composition of pantothenate that includes culturing a microorganism having at least one pantothenate biosynthetic enzyme deregulated (*e.g.*, deregulated such that pantothenate production is enhanced), said enzyme being selected, for example, from the group consisting of PanB (or ketopantoate hydroxymethyltransferase), PanC (or pantothenate synthetase), PanD (or aspartate-α-decarboxylase), PanE1 (or ketopantoate reductase). In another embodiment, the invention features a process for the production of a HMBPA-free composition of pantothenate that includes culturing a microorganism having at least two pantothenate biosynthetic enzymes deregulated, said enzymes being selected, for example, from the group consisting of PanB (or ketopantoate hydroxymethyltransferase), PanC (or pantothenate synthetase), PanD (or aspartate-α-decarboxylase), and PanE1 (or ketopantoate reductase). In another embodiment, the invention features a process for the production of a HMBPA-free composition of pantothenate that includes culturing a microorganism having at least three pantothenate biosynthetic enzymes deregulated, said enzymes being selected, for example, from the group consisting of PanB (or ketopantoate hydroxymethyltransferase), PanC (or pantothenate synthetase), PanD (or aspartate-α-decarboxylase), and PanE1 (or ketopantoate reductase). In another embodiment, the invention features a process for the production of a HMBPA-free composition of pantothenate that includes culturing a microorganism having at least four pantothenate biosynthetic enzymes deregulated, for example, a microorganism having PanB (or ketopantoate hydroxymethyltransferase), PanC (or pantothenate synthetase), PanD (or aspartate-α-decarboxylase), and PanE1 (or ketopantoate reductase) deregulated.

In another aspect, the invention features a process for the production of a HMBPA-free pantothenate that includes culturing a microorganism having a deregulated pantothenate biosynthetic pathway under conditions such that a HMBPA-free pantothenate composition is produced, the microorganism further having a deregulated isoleucine-valine biosynthetic pathway. The term "isoleucine-valine biosynthetic pathway" includes the biosynthetic pathway involving isoleucine-valine biosynthetic enzymes (*e.g.*, polypeptides encoded by biosynthetic enzyme-encoding genes), compounds (*e.g.*, substrates, intermediates or products), cofactors and the like utilized in the formation or synthesis of conversion of pyruvate to valine or isoleucine. The term "isoleucine-valine biosynthetic pathway" includes the biosynthetic pathway leading to

the synthesis of valine or isoleucine in microorganisms (*e.g.*, *in vivo*) as well as the biosynthetic pathway leading to the synthesis of valine or isoleucine *in vitro*.

As used herein, a microorganism "having a deregulated isoleucine-valine (*ilv*) pathway" includes a microorganism having at least one isoleucine-valine (*ilv*) biosynthetic enzyme deregulated (*e.g.*, overexpressed) (both terms as defined herein) such that isoleucine and/or valine and/or the valine precursor, α-ketoisovaerate (α-KIV) production is enhanced (*e.g.*, as compared to isoleucine and/or valine and/or α-KIV production in said microorganism prior to deregulation of said biosynthetic enzyme or as compared to a wild-type microorganism). Figure 1 includes a schematic representation of the isoleucine-valine biosynthetic pathway. Isoleucine-valine biosynthetic enzymes are depicted in bold italics and their corresponding genes indicated in italics. The term "isoleucine-valine biosynthetic enzyme" includes any enzyme utilized in the formation of a compound (*e.g.*, intermediate or product) of the isoleucine-valine biosynthetic pathway. According to Figure 1, synthesis of valine from pyruvate proceeds *via* the intermediates, acetolactate, α,β-dihydroxyisovalerate (α,β-DHIV) and α-ketoisovalerate (α-KIV). Formation of acetolactate from pyruvate is catalyzed by the isoleucine-valine biosynthetic enzyme acetohydroxyacid synthetase (the *ilvBN* gene products, or alternatively, the *alsS* gene product). Formation of α,β-DHIV from acetolactate is catalyzed by the isoleucine-valine biosynthetic enzyme acetohydroxyacid isomeroreductase (the *ilvC* gene product). Synthesis of α-KIV from α,β-DHIV is catalyzed by the isoleucine-valine biosynthetic enzyme dihydroxyacid dehydratase (the *ilvD* gene product). Moreover, valine and isoleucine can be interconverted with their respective α-keto compounds by branched chain amino acid transaminases. Isoleucine-valine biosynthetic enzymes may also perform an alternative function as enzymes in the HMBPA biosynthetic pathway described herein.

Accordingly, in one embodient, the invention features a process for the production of a HMBPA-free composition of pantothenate that includes culturing a microorganism having at least one isoleucine-valine (*ilv*) biosynthetic enzyme deregulated (*e.g.*, deregulated such that valine and/or isoleucine and/or α-KIV production is enhanced), said enzyme being selected, for example, from the group consisting of IlvBN, AlsS (or acetohydroxyacid synthetase), IlvC (or acetohydroxyacid isomeroreductase) and IlvD (or dihydroxyacid dehydratase). In another embodiment, the invention features a process for the production of a HMBPA-free composition of pantothenate that includes culturing a microorganism having at least two isoleucine-valine (*ilv*) biosynthetic enzymes deregulated, said enzyme being selected, for example, from the group consisting of IlvBN, AlsS (or acetohydroxyacid synthetase), IlvC (or

acetohydroxyacid isomeroreductase) and IlvD (or dihydroxyacid dehydratase). In another embodiment, the invention features a process for the production of a HMBPA-free composition of pantothenate that includes culturing a microorganism having at least three isoleucine-valine (*ilv*) biosynthetic enzymes deregulated, for example, said microorganism having IlvBN or AlsS (or acetohydroxyacid synthetase), IlvC (or acetohydroxyacid isomeroreductase) and IlvD (or dihydroxyacid dehydratase) deregulated.

As mentioned herein, enzymes of the pantothenate biosynthetic pathway and/or the isoleucine-valine (*ilv*) pathway have been discovered to have an alternative activity in the synthesis of [R]-3-(2-hydroxy-3-methyl-butyrylamino)-propionic acid ("HMBPA") or the [R]-3-(2-hydroxy-3-methyl-butyrylamino)-propionic acid ("HMBPA") biosynthetic pathway. The term "[R]-3-(2-hydroxy-3-methyl-butyrylamino)-propionic acid ("HMBPA") biosynthetic pathway" includes the alternative biosynthetic pathway involving biosynthetic enzymes and compounds (*e.g.*, substrates and the like) traditionally associated with the pantothenate biosynthetic pathway and/or isoleucine-valine (*ilv*) biosynthetic pathway utilized in the formation or synthesis of HMBPA. The term "HMBPA biosynthetic pathway" includes the biosynthetic pathway leading to the synthesis of HMBPA in microorganisms (*e.g.*, *in vivo*) as well as the biosynthetic pathway leading to the synthesis of HMBPA *in vitro*.

The term "HMBPA biosynthetic enzyme" includes any enzyme utilized in the formation of a compound (*e.g.*, intermediate or product) of the HMBPA biosynthetic pathway. For example, synthesis of 2-hydroxyisovaleric acid (α-HIV) from α-ketoisovalerate (α-KIV) is catalyzed by the *panE1* or *panE2* gene product (PanE1 is alternatively referred to herein as ketopantoate reductase) and/or is catalyzed by the *ilvC* gene product (alternatively referred to herein as acetohydroxyacid isomeroreductase). Formation of HMBPA from β-alanine and α-HIV is catalyzed by the *panC* gene product (alternatively referred to herein as pantothenate synthetase).

The term "[R]-3-(2-hydroxy-3-methyl-butyrylamino)-propionic acid ("HMBPA")" includes the free acid form of HMBPA, also referred to as "[R]-3-(2-hydroxy-3-methyl-butyrylamino)-propionate" as well as any salt thereof (*e.g.*, derived by replacing the acidic hydrogen of 3-(2-hydroxy-3-methyl-butyrylamino)-propionic acid or 3-(2-hydroxy-3-methyl-butyrylamino)-propionate with a cation, for example, calcium, sodium, potassium, ammonium), also referred to as a "3-(2-hydroxy-3-methyl-butyrylamino)-propionic acid salt" or "HMBPA salt". Preferred HMBPA salts are calcium HMBPA or sodium HMBPA. HMBPA salts of the present invention include salts prepared *via* conventional methods from the free acids described herein. An

HMBPA salt of the present invention can likewise be converted to a free acid form of 3-(2-hydroxy-3-methyl-butyrylamino)-propionic acid or 3-(2-hydroxy-3-methyl-butyrylamino)-propionate by conventional methodology.

Based at least in part on the discovery that overexpression or deregulation of the pantothenate biosynthetic pathway and/or isoleucine-valine (*ilv*) pathway can result in alternative production of HMBPA (as compared to desired pantothenate production), the present invention features processes for the production of HMBPA-free pantothenate that involve culturing microorganisms that not only have the pantothenate biosynthetic pathway and/or the isoleucine-valine (*ilv*) pathway deregulated, but that further have certain enzymes selectively regulated such that production of HMBPA-free pantothenate compositions is favored. As defined herein, the term "selectively regulated" includes selecting for regulation or targeting a particular enzyme or enzymes from the pantothenate biosynthetic pathway or the isoleucine-valine (*ilv*) pathway known to be involved in both pantothenate and HMBPA synthesis in a manner that favors pantothenate production over HMBPA production. Preferred enzymes selected or targeted for regulation include PanE1, PanE2, PanB and/or IlvC.

In one embodiment, Pan E1 is selectively regulated. For example, the present inventors have discovered that overexpression of PanE1 can catalyze HMBPA precursor formation, therefore selectively regulating the amount or activity (*e.g.*, to slightly decrease PanE1 levels or activity) can shift formation from HMBPA production to pantothenate production (*i.e.*, favor pantothenate production over HMBPA production). Moreover, it has been discovered that PanE2 favors HMBPA production. Accordingly, another embodiment, features deleting or regulating *panE2*.

Likewise, the present inventors have discovered that increasing PanB activity can shift formation from the alternative HMBPA biosynthetic pathway to the pantothenate biosynthetic pathway, therefore selectively regulating the amount or activity of PanB can favor pantothenate production over HMBPA production. In one embodiment, PanB activity is increased by overexpressing or deregulating the *panB* gene. In another embodiment, PanB activity is increased by expressing multiple copies of the *panB* gene. PanB activity can be increased by decreasing feedback inhibtion of PanB. In particular, is has been discovered that PanB activity can be increased by regulating (*e.g.*, selectively regulating) pantothenate kinase, a key enzyme in the formation of Coenzyme A (CoA) from pantothenate (see *e.g.*, U.S. Patent Application Serial No. 09/09/667,569). Regulation of pantothenate kinase (*e.g.*, decreasing the

activity or level of pantothenate kinase) reduced the production of CoA, in turn reducing feedback inhibition of PanB as well as favoring pantothenate accumulation. In one embodiment, pantotheante kinase activity is decreased (and PanB activity is in turn increased) by deleting CoaA and downregulating CoaX activity (CoaA and CoaX are both capable of catalyzing the first step in CoA biosynthesis in certain microorganisms). In another embodiment, pantothenate kinase activity is decreased (and PanB activity is in turn increased) by deleting CoaX and downregulating CoaA. In yet another embodiment, pantotheante kinase activity is decreased (and PanB activity is in turn increased) by downregulating CoaA and CoaX activities.

Yet another aspect of the present invention features processes for the production of HMBPA-free pantothenate that include culturing mocroorganisms under culture conditions selected to favor pantothenate production over HMBPA production. In particular, it has been discovered that conditions including, but not limited to, reduced steady state glucose, increased steady state dissolved oxygen and/or excess serine favor pantothenate production over HMBPA production. The term "reduced steady state glucose" includes steady state glucose levels less or lower that those routinely utilized for culturing the microorganism in question. For example, culturing the *Bacillus* microorganisms described in the instant Examples is routinely done in the presence of about 0.2-1.0 g/L steady state glucose. Accordingly, reduced steady state glucose levels preferably include levels of less than 0.2 g.L steady state glucose. The term "increased steady state dissolved oxygen" includes steady state dissolved oxygen levels increased or higher than those routinely utilized for culturing the microorganism in question and, for example, inversely correlates with reduced steady state glucose levels. For example, culturing the *Bacillus* microorganisms described in the instant Examples is routinely done in the presence of about 10-30% dissolved oxygen. Accordingly, increased steady state dissolved oxygen can include levels of greater that 30% dissolved oxygen, preferably as great as 95% dissolved oxygen. The term "excess serine" includes serine levels increased or higher that those routinely utilized for culturing the microorganism in question. For example, culturing the *Bacillus* microorganisms described in the instant Examples is routinely done in the presence of about 0-2.5 g/L serine. Accordingly, excess serine levels can include levels of greater than 2.5 g/L serine, preferably between about 2.5 and 20 g/L serine.

In yet another embodiment, HMBPA production is favored by increasing pantothenate and/or isoleucine-valine (*ilv*) biosynthetic pathway precursors and/or intermediates as defined herein (*e.g.*, culturing microorganisms in the presence of excess

β-alanine, valine and/or α-KIV) or, alternatively, culturing microorganisms capable of producing significant levels of β-alanine in the absence of a β-alanine feed (*i.e.*, β-alanine independent microorganisms, as described in U.S. Patent Application Serial No. 09/09/667,569).

Various aspects of the invention are described in further detail in the following subsections.

I. Targeting Genes Encoding Various Pantothenate and/or Isoleucine-Valine(ilv) and/or HMBPA Biosynthetic Enzymes

In one embodiment, the present invention features targeting or modifying various biosynthetic enzymes of the pantothenate and/or isoleucine-valine(*ilv*) and/or HMBPA biosynthetic pathways. In particular, the invention features modifying various enzymatic activities associated with said pathways by modifying or altering the genes encoding said biosynthetic enzymes.

The term "gene", as used herein, includes a nucleic acid molecule (*e.g.*, a DNA molecule or segment thereof) that, in an organism, can be separated from another gene or other genes, by intergenic DNA (*i.e.*, intervening or spacer DNA which naturally flanks the gene and/or separates genes in the chromosomal DNA of the organism). Alternatively, a gene may slightly overlap another gene (*e.g.*, the 3' end of a first gene overlapping the 5' end of a second gene), the overlapping genes separated from other genes by intergenic DNA. A gene may direct synthesis of an enzyme or other protein molecule (*e.g.*, may comprise coding seqeunces, for example, a contiguous open reading frame (ORF) which encodes a protein) or may itself be functional in the organism. A gene in an organism, may be clustered in an operon, as defined herein, said operon being separated from other genes and/or operons by the intergenic DNA. An "isolated gene", as used herein, includes a gene which is essentially free of sequences which naturally flank the gene in the chromosomal DNA of the organism from which the gene is derived (*i.e.*, is free of adjacent coding sequences that encode a second or distinct protein, adjacent structural sequences or the like) and optionally includes 5' and 3' regulatory sequences, for example promoter sequences and/or terminator sequences. In one embodiment, an isolated gene includes predominantly coding sequences for a protein (*e.g.*, sequences which encode *Bacillus* proteins). In another embodiment, an isolated gene includes coding sequences for a protein (*e.g.*, for a *Bacillus* protein) and adjacent 5' and/or 3' regulatory sequences from the chromosomal DNA of the organism from which

the gene is derived (*e.g.*, adjacent 5' and/or 3' *Bacillus* regulatory sequences). Preferably, an isolated gene contains less than about 10 kb, 5 kb, 2 kb, 1 kb, 0.5 kb, 0.2 kb, 0.1 kb, 50 bp, 25 bp or 10 bp of nucleotide sequences which naturally flank the gene in the chromosomal DNA of the organism from which the gene is derived.

The term "operon" includes at least two adjacent genes or ORFs, optionally overlapping in sequence at either the 5' or 3' end of at least one gene or ORF. The term "operon" includes a coordinated unit of gene expression that contains a promoter and possibly a regulatory element associated with one or more adjacent genes or ORFs (*e.g.*, structural genes encoding enzymes, for example, biosynthetic enzymes). Expression of the genes (*e.g.*, structural genes) can be coordinately regulated, for example, by regulatory proteins binding to the regulatory element or by anti-termination of transcription. The genes of an operon (*e.g.*, structural genes) can be transcribed to give a single mRNA that encodes all of the proteins.

A "gene having a mutation" or "mutant gene" as used herein, includes a gene having a nucleotide sequence which includes at least one alteration (*e.g.*, substitution, insertion, deletion) such that the polypeptide or protein encoded by said mutant exhibits an activity that differs from the polypeptide or protein encoded by the wild-type nucleic acid molecule or gene. In one embodiment, a gene having a mutation or mutant gene encodes a polypeptide or protein having an increased activity as compared to the polypeptide or protein encoded by the wild-type gene, for example, when assayed under similar conditions (*e.g.*, assayed in microorganisms cultured at the same temperature). As used herein, an "increased activity" or "increased enzymatic activity" is one that is at least 5% greater than that of the polypeptide or protein encoded by the wild-type nucleic acid molecule or gene, preferably at least 5-10% greater, more preferably at least 10-25% greater and even more preferably at least 25-50%, 50-75% or 75-100% greater than that of the polypeptide or protein encoded by the wild-type nucleic acid molecule or gene. Ranges intermediate to the above-recited values, *e.g.*, 75-85%, 85-90%, 90-95%, are also intended to be encompassed by the present invention. As used herein, an "increased activity" or "increased enzymatic activity" can also include an activity that is at least 1.25-fold greater than the activity of the polypeptide or protein encoded by the wild-type gene, preferably at least 1.5-fold greater, more preferably at least 2-fold greater and even more preferably at least 3-fold, 4-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold greater than the activity of the polypeptide or protein encoded by the wild-type gene.

In another embodiment, a gene having a mutation or mutant gene encodes a polypeptide or protein having a reduced activity as compared to the polypeptide or protein encoded by the wild-type gene, for example, when assayed under similar conditions (*e.g.*, assayed in microorganisms cultured at the same temperature). A mutant gene also can encode no polypeptide or have a reduced level of production of the wild-type polypeptide. As used herein, a "reduced activity" or "reduced enzymatic activity" is one that is at least 5% less than that of the polypeptide or protein encoded by the wild-type nucleic acid molecule or gene, preferably at least 5-10% less, more preferably at least 10-25% less and even more preferably at least 25-50%, 50-75% or 75-100% less than that of the polypeptide or protein encoded by the wild-type nucleic acid molecule or gene. Ranges intermediate to the above-recited values, *e.g.*, 75-85%, 85-90%, 90-95%, are also intended to be encompassed by the present invention. As used herein, a "reduced activity" or "reduced enzymatic activity" can also include an activity that has been deleted or "knocked out" (*e.g.*, approximately 100% less activity than that of the polypeptide or protein encoded by the wild-type nucleic acid molecule or gene).

Activity can be determined according to any well accepted assay for measuring activity of a particular protein of interest. Activity can be measured or assayed directly, for example, measuring an activity of a protein isolated or purified from a cell or microorganism. Alternatively, an activity can be measured or assayed within a cell or microorganism or in an extracellular medium. For example, assaying for a mutant gene (*i.e.*, said mutant encoding a reduced enzymatic activity) can be accomplished by expressing the mutated gene in a microorganism, for example, a mutant microorganism in which the enzyme is a temperature-sensitive, and assaying the mutant gene for the ability to complement a temperature sensitive (Ts) mutant for enzymatic activity. A mutant gene that encodes an "increased enzymatic activity" can be one that complements the Ts mutant more effectively than, for example, a corresponding wild-type gene. A mutant gene that encodes a "reduced enzymatic activity" is one that complements the Ts mutant less effectively than, for example, a corresponding wild-type gene.

It will be appreciated by the skilled artisan that even a single substitution in a nucleic acid or gene sequence (*e.g.*, a base substitution that encodes an amino acid change in the corresponding amino acid sequence) can dramatically affect the activity of an encoded polypeptide or protein as compared to the corresponding wild-type polypeptide or protein. A mutant gene (*e.g.*, encoding a mutant polypeptide or protein), as defined herein, is readily distinguishable from a nucleic acid or gene encoding a protein homologue in that a mutant gene encodes a protein or polypeptide having an

altered activity, optionally observable as a different or distinct phenotype in a microorganism expressing said mutant gene or producing said mutant protein or polypeptide (*i.e.*, a mutant microorganism) as compared to a corresponding microorganism expressing the wild-type gene. By contrast, a protein homologue can have an identical or substantially similar activity, optionally phenotypically indiscernable when produced in a microorganism, as compared to a corresponding microorganism expressing the wild-type gene. Accordingly it is not, for example, the degree of sequence identity between nucleic acid molecules, genes, protein or polypeptides that serves to distinguish between homologues and mutants, rather it is the activity of the encoded protein or polypeptide that distinguishes between homologues and mutants: homologues having, for example, low (*e.g.*, 30-50% sequence identity) sequence identity yet having substantially equivalent functional activities, and mutants, for example sharing 99% sequence identity yet having dramatically different or altered functional activities.

It will also be appreciated by the skilled artisan that nucleic acid molecules, genes, protein or polypeptides for use in the instant invention can be derived from any microorganisms having a HMBPA biosynthetic pathway, an *ilv* biosynthetic pathway or a pantothenate biosynthetic pathway. Such nucleic acid molecules, genes, protein or polypeptides can be identified by the skilled artisan using known techniques such as homology screening, sequence comparison and the like, and can be modified by the skilled artisan in such a way that expression or production of these nucleic acid molecules, genes, protein or polypeptides occurs in a recombinant microorganism (*e.g.*, by using appropriate promotors, ribosomal binding sites, expression or integration vectors, modifying the sequence of the genes such that the transcription is increased (taking into account the preferable codon usage), etc., according to techniques described herein and those known in the art).

In one embodiment, the genes of the present invention are derived from a Gram positive microorganism organism (*e.g.*, a microorganism which retains basic dye, for example, crystal violet, due to the presence of a Gram-positive wall surrounding the microorganism). The term "derived from" (*e.g.*, "derived from" a Gram positive microorganism) refers to a gene which is naturally found in the microorganism (*e.g.*, is naturally found in a Gram positive microorganism). In a preferred embodiment, the genes of the present invention are derived from a microorganism belonging to a genus selected from the group consisting of *Bacillus*, *Cornyebacterium* (*e.g.*, *Cornyebacterium glutamicum*), *Lactobacillus*, *Lactococci* and *Streptomyces*. In a more preferred embodiment, the genes of the present invention are derived from a microorganism is of

the genus *Bacillus*. In another preferred embodiment, the genes of the present invention are derived from a microorganism selected from the group consisting of *Bacillus subtilis, Bacillus lentimorbus, Bacillus lentus, Bacillus firmus, Bacillus pantothenticus, Bacillus amyloliquefaciens, Bacillus cereus, Bacillus circulans, Bacillus coagulans, Bacillus licheniformis, Bacillus megaterium, Bacillus pumilus, Bacillus thuringiensis, Bacillus halodurans*, and other Group 1 *Bacillus* species, for example, as characterized by 16S rRNA type. In another preferred embodiment, the gene is derived from *Bacillus brevis* or *Bacillus stearothermophilus*. In another preferred embodiment, the genes of the present invention are derived from a microorganism selected from the group consisting of *Bacillus licheniformis, Bacillus amyloliquefaciens, Bacillus subtilis*, and *Bacillus pumilus*. In a particularly preferred embodiment, the gene is derived from *Bacillus subtilis* (*e.g.*, is *Bacillus subtilis*-derived). The term "derived from *Bacillus subtilis*" or "*Bacillus subtilis*-derived" includes a gene which is naturally found in the microorganism *Bacillus subtilis*. Included within the scope of the present invention are *Bacillus*-derived genes (*e.g.*, *B. subtilis*-derived genes), for example, *Bacillus* or *B. subtilis coaX* genes, *serA* genes, *glyA* genes, *coaA* genes, *pan* genes and/or *ilv* genes.

In another embodiment, the genes of the present invention are derived from a Gram negative (excludes basic dye) microorganism. In a preferred embodiment, the genes of the present invention are derived from a microorganism belonging to a genus selected from the group consisting of *Salmonella* (*e.g.*, *Salmonella typhimurium*), *Escherichia, Klebsiella, Serratia,* and *Proteus*. In a more preferred embodiment, the genes of the present invention are derived from a microorganism of the genus *Escherichia*. In an even more preferred embodiment, the genes of the present invention are derived from *Escherichia coli*. In another embodiment, the genes of the present invention are derived from *Saccharomyces* (*e.g.*, *Saccharomyces cerevisiae*).

II. Recombinant Nucleic Acid Molecules and Vectors

The present invention further features recombinant nucleic acid molecules (*e.g.*, recombinant DNA molecules) that include genes described herein (*e.g.*, isolated genes), preferably *Bacillus* genes, more preferably *Bacillus subtilis* genes, even more preferably *Bacillus subtilis* pantothenate biosynthetic genes and/or isoleucine-valine (*ilv*) biosynthetic genes and/or HMBPA biosynthetic genes. The term "recombinant nucleic acid molecule" includes a nucleic acid molecule (*e.g.*, a DNA molecule) that has been altered, modified or engineered such that it differs in nucleotide sequence from the native or natural nucleic acid molecule from which the recombinant nucleic acid molecule was derived (*e.g.*, by addition, deletion or substitution of one or

more nucleotides). Preferably, a recombinant nucleic acid molecule (*e.g.*, a recombinant DNA molecule) includes an isolated gene of the present invention operably linked to regulatory sequences. The phrase "operably linked to regulatory sequence(s)" means that the nucleotide sequence of the gene of interest is linked to the regulatory sequence(s) in a manner which allows for expression (*e.g.*, enhanced, increased, constitutive, basal, attenuated, decreased or repressed expression) of the gene, preferably expression of a gene product encoded by the gene (*e.g.*, when the recombinant nucleic acid molecule is included in a recombinant vector, as defined herein, and is introduced into a microorganism).

The term "regulatory sequence" includes nucleic acid sequences which affect (*e.g.*, modulate or regulate) expression of other nucleic acid sequences (*i.e.*, genes). In one embodiment, a regulatory sequence is included in a recombinant nucleic acid molecule in a similar or identical position and/or orientation relative to a particular gene of interest as is observed for the regulatory sequence and gene of interest as it appears in nature, *e.g.*, in a native position and/or orientation. For example, a gene of interest can be included in a recombinant nucleic acid molecule operably linked to a regulatory sequence which accompanies or is adjacent to the gene of interest in the natural organism (*e.g.*, operably linked to "native" regulatory sequences (*e.g.*, to the "native" promoter). Alternatively, a gene of interest can be included in a recombinant nucleic acid molecule operably linked to a regulatory sequence which accompanies or is adjacent to another (*e.g.*, a different) gene in the natural organism. Alternatively, a gene of interest can be included in a recombinant nucleic acid molecule operably linked to a regulatory sequence from another organism. For example, regulatory sequences from other microbes (*e.g.*, other bacterial regulatory sequences, bacteriophage regulatory sequences and the like) can be operably linked to a particular gene of interest.

In one embodiment, a regulatory sequence is a non-native or non-naturally-occurring sequence (*e.g.*, a sequence which has been modified, mutated, substituted, derivatized, deleted including sequences which are chemically synthesized). Preferred regulatory sequences include promoters, enhancers, termination signals, anti-termination signals and other expression control elements (*e.g.*, sequences to which repressors or inducers bind and/or binding sites for transcriptional and/or translational regulatory proteins, for example, in the transcribed mRNA). Such regulatory sequences are described, for example, in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989. Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in a microorganism

(*e.g.*, constitutive promoters and strong constitutive promoters), those which direct inducible expression of a nucleotide sequence in a microorganism (*e.g.*, inducible promoters, for example, xylose inducible promoters) and those which attenuate or repress expression of a nucleotide sequence in a microorganism (*e.g.*, attenuation signals or repressor sequences). It is also within the scope of the present invention to regulate expression of a gene of interest by removing or deleting regulatory sequences. For example, sequences involved in the negative regulation of transcription can be removed such that expression of a gene of interest is enhanced.

In one embodiment, a recombinant nucleic acid molecule of the present invention includes a nucleic acid sequence or gene that encodes at least one bacterial gene product (*e.g.*, a pantothenate biosynthetic enzyme, an isoleucine-valine biosynthetic enzyme and/or a HMBPA biosynthetic enzyme) operably linked to a promoter or promoter sequence. Preferred promoters of the present invention include *Bacillus* promoters and/or bacteriophage promoters (*e.g.*, bacteriophage which infect *Bacillus*). In one embodiment, a promoter is a *Bacillus* promoter, preferably a strong *Bacillus* promoter (*e.g.*, a promoter associated with a biochemical housekeeping gene in *Bacillus* or a promoter associated with a glycolytic pathway gene in *Bacillus*). In another embodiment, a promoter is a bacteriophage promoter. In a preferred embodiment, the promoter is from the bacteriophage SP01. In a particularly preferred embodiment, a promoter is selected from the group consisting of P_{15}, P_{26} or P_{veg}, having for example, the following respective seqeunces:

GCTATTGACGACAGCTATGGTTCACTGTCCACCAACCAAAACTGTGCTCAGT
ACCGCCAATATTTCTCCCTTGAGGGGTACAAAGAGGTGTCCCTAGAAGAGAT
CCACGCTGTGTAAAAATTTTACAAAAAGGTATTGACTTTCCCTACAGGGTGT
GTAATAATTTAATTACAGGCGGGGGCAACCCCGCCTGT(SEQ ID NO:1),

GCCTACCTAGCTTCCAAGAAAGATATCCTAACAGCACAAGAGCGGAAAGAT
GTTTTGTTCTACATCCAGAACAACCTCTGCTAAAATTCCTGAAAAATTTTGCA
AAAAGTTGTTGACTTTATCTACAAGGTGTGGTATAATAATCTTAACAACAGC
AGGACGC (SEQ ID NO:2), and

GAGGAATCATAGAATTTTGTCAAAATAATTTTATTGACAACGTCTTATTAAC
GTTGATATAATTTAAATTTTATTTGACAAAAATGGGCTCGTGTTGTACAATA
AATGTAGTGAGGTGGATGCAATG (SEQ ID NO:3). Additional preferred promoters include *tef* (the translational elongation factor (TEF) promoter) and *pyc* (the pyruvate carboxylase (PYC) promoter), which promote high level expression in *Bacillus* (*e.g.*, *Bacillus subtilis*). Additional preferred promoters, for example, for use in Gram positive microorganisms include, but are not limited to, *amy* and SPO2 promoters. Additional

preferred promoters, for example, for use in Gram negative microorganisms include, but are not limited to, *cos*, *tac*, *trp*, *tet*, *trp-tet*, *lpp*, *lac*, *lpp-lac*, *lacIQ*, T7, T5, T3, *gal*, *trc*, *ara*, SP6, λ-PR or λ-PL.

In another embodiment, a recombinant nucleic acid molecule of the present invention includes a terminator sequence or terminator sequences (*e.g.*, transcription terminator sequences). The term "terminator sequences" includes regulatory sequences that serve to terminate transcription of mRNA. Terminator sequences (or tandem transcription terminators) can further serve to stabilize mRNA (*e.g.*, by adding structure to mRNA), for example, against nucleases.

In yet another embodiment, a recombinant nucleic acid molecule of the present invention includes sequences that allow for detection of the vector containing said sequences (*i.e.*, detectable and/or selectable markers), for example, genes that encode antibiotic resistance sequences or that overcome auxotrophic mutations, for example, *trpC*, drug markers, fluorescent markers, and/or colorimetric markers (*e.g.*, *lacZ*/β-galactosidase). In yet another embodiment, a recombinant nucleic acid molecule of the present invention includes an artificial ribosome binding site (RBS) or a sequence that gets transcribed into an artificial RBS. The term "artificial ribosome binding site (RBS)" includes a site within an mRNA molecule (*e.g.*, coded within DNA) to which a ribosome binds (*e.g.*, to initiate translation) which differs from a native RBS (*e.g.*, a RBS found in a naturally-occurring gene) by at least one nucleotide. Preferred artificial RBSs include about 5-6, 7-8, 9-10, 11-12, 13-14, 15-16, 17-18, 19-20, 21-22, 23-24, 25-26, 27-28, 29-30 or more nucleotides of which about 1-2, 3-4, 5-6, 7-8, 9-10, 11-12, 13-15 or more differ from the native RBS (*e.g.*, the native RBS of a gene of interest, for example, the native *panB* RBS TAAACATGAGGAGGAGAAAACATG (SEQ ID NO:4) or the native *panD* RBS ATTCGAGAAATGGAGAGAATATAATATG (SEQ ID NO:5)). Preferably, nucleotides that differ are substituted such that they are identical to one or more nucleotides of an ideal RBS when optimally aligned for comparisons. Ideal RBSs include, but are not limited to, AGAAAGGAGGTGA (SEQ ID NO:6), TTAAGAAAGGAGGTGANNNNATG (SEQ ID NO:7), TTAGAAAGGAGGTGANNNNNATG (SEQ ID NO:8), AGAAAGGAGGTGANNNNNNNATG (SEQ ID NO:9), and AGAAAGGAGGTGANNNNNNATG (SEQ ID NO:10). Artificial RBSs can be used to replace the naturally-occurring or native RBSs associated with a particular gene. Artificial RBSs preferably increase translation of a particular gene. Preferred artificial RBSs (*e.g.*, RBSs for increasing the translation of *panB*, for example, of *B. subtilis panB*) include CCCTCTAGAAGGAGGAGAAAACATG (SEQ ID NO:11) and

CCCTCTAGAGGAGGAGAAAACATG (SEQ ID NO:12). Preferred artificial RBSs (e.g., RBSs for increasing the translation of *panD*, for example, of *B. subtilis panD*) include TTAGAAAGGAGGATTTAAATATG (SEQ ID NO:13), TTAGAAAGGAGGTTTAATTAATG (SEQ ID NO:14), TTAGAAAGGAGGTGATTTAAATG (SEQ ID NO:15), TTAGAAAGGAGGTGTTTAAAATG (SEQ ID NO:16), ATTCGAGAAAGGAGG TGAATATAATATG (SEQ ID NO:17), ATTCGAGAAAGGAGGTGAATAATAATG (SEQ ID NO:18), and ATTCGTAGAAAGGAGGTGAATTAATATG (SEQ ID NO:19).

The present invention further features vectors (*e.g.*, recombinant vectors) that include nucleic acid molecules (*e.g.*, genes or recombinant nucleic acid molecules comprising said genes) as described herein. The term "recombinant vector" includes a vector (*e.g.*, plasmid, phage, phasmid, virus, cosmid or other purified nucleic acid vector) that has been altered, modified or engineered such that it contains greater, fewer or different nucleic acid sequences than those included in the native or natural nucleic acid molecule from which the recombinant vector was derived. Preferably, the recombinant vector includes a biosynthetic enzyme-encoding gene or recombinant nucleic acid molecule including said gene, operably linked to regulatory sequences, for example, promoter sequences, terminator sequences and/or artificial ribosome binding sites (RBSs), as defined herein. In another embodiment, a recombinant vector of the present invention includes sequences that enhance replication in bacteria (*e.g.*, replication-enhancing sequences). In one embodiment, replication-enhancing sequences function in *E. coli*. In another embodiment, replication-enhancing sequences are derived from pBR322.

In yet another embodiment, a recombinant vector of the present invention includes antibiotic resistance sequences. The term "antibiotic resistance sequences" includes sequences which promote or confer resistance to antibiotics on the host organism (*e.g.*, *Bacillus*). In one embodiment, the antibiotic resistance sequences are selected from the group consisting of *cat* (chloramphenicol resistance) sequences, *tet* (tetracycline resistance) sequences, *erm* (erythromycin resistance) sequences, *neo* (neomycin resistance) sequences, *kan* (kanamycin resistence) sequences and *spec* (spectinomycin resistance) sequences. Recombinant vectors of the present invention can further include homologous recombination sequences (*e.g.*, sequences designed to allow recombination of the gene of interest into the chromosome of the host organism). For example, *bpr*, *vpr*, or *amyE* sequences can be used as homology targets for recombination into the host chromosome. It will further be appreciated by one of skill in the art that the design of a vector can be tailored depending on such factors as the choice

of microorganism to be genetically engineered, the level of expression of gene product desired and the like.

IV. Recombinant Microorganisms

The present invention further features microorganisms, *i.e.*, recombinant microorganisms, that include vectors or genes (*e.g.*, wild-type and/or mutated genes) as described herein. As used herein, the term "recombinant microorganism" includes a microorganism (*e.g.*, bacteria, yeast cell, fungal cell, etc.) that has been genetically altered, modified or engineered (*e.g.*, genetically engineered) such that it exhibits an altered, modified or different genotype and/or phenotype (*e.g.*, when the genetic modification affects coding nucleic acid sequences of the microorganism) as compared to the naturally-occurring microorganism from which it was derived.

In one embodiment, a recombinant microorganism of the present invention is a Gram positive organism (*e.g.*, a microorganism which retains basic dye, for example, crystal violet, due to the presence of a Gram-positive wall surrounding the microorganism). In a preferred embodiment, the recombinant microorganism is a microorganism belonging to a genus selected from the group consisting of *Bacillus*, *Cornyebacterium*, *Lactobacillus*, *Lactococci* and *Streptomyces*. In a more preferred embodiment, the recombinant microorganism is of the genus *Bacillus*. In another preferred embodiment, the recombinant microorganism is selected from the group consisting of *Bacillus subtilis, Bacillus lentimorbus*, *Bacillus lentus*, *Bacillus firmus*, *Bacillus pantothenticus*, *Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus circulans*, *Bacillus coagulans*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus thuringiensis*, *Bacillus halodurans*, and other Group 1 *Bacillus* species, for example, as characterized by 16S rRNA type. In another preferred embodiment, the recombinant microorganism is *Bacillus brevis* or *Bacillus stearothermophilus*. In another preferred embodiment, the recombinant microorganism is selected from the group consisting of *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, and *Bacillus pumilus*.

In another embodiment, the recombinant microorganism is a Gram negative (excludes basic dye) organism. In a preferred embodiment, the recombinant microorganism is a microorganism belonging to a genus selected from the group consisting of *Salmonella, Escherichia, Klebsiella, Serratia,* and *Proteus*. In a more preferred embodiment, the recombinant microorganism is of the genus *Escherichia*. In an even more preferred embodiment, the recombinant microorganism is *Escherichia*

coli. In another embodiment, the recombinant microorganism is *Saccharomyces* (*e.g.*, *S. cerevisiae*).

A preferred "recombinant" microorganism of the present invention is a microorganism having a deregulated pantothenate biosynthesis pathway or enzyme, a deregulated isoleucine-valine (*ilv*) biosynthetic pathway or enzyme and/or a modified HMBPA biosynthetic pathway or enzyme. The term "deregulated" or "deregulation" includes the alteration or modification of at least one gene in a microorganism that encodes an enzyme in a biosynthetic pathway, such that the level or activity of the biosynthetic enzyme in the microorganism is altered or modified. Preferably, at least one gene that encodes an enzyme in a biosynthetic pathway is altered or modified such that the gene product is enhanced or increased. The phrase "deregulated pathway" can also include a biosynthetic pathway in which more than one gene that encodes an enzyme in a biosynthetic pathway is altered or modified such that the level or activity of more than one biosynthetic enzyme is altered or modified. The ability to "deregulate" a pathway (*e.g.*, to simultaneously deregulate more than one gene in a given biosynthetic pathway) in a microorganism in some cases arises from the particular phenomenon of microorganisms in which more than one enzyme (*e.g.*, two or three biosynthetic enzymes) are encoded by genes occurring adjacent to one another on a contiguous piece of genetic material termed an "operon" (defined herein). Due to the coordinated regulation of genes included in an operon, alteration or modification of the single promoter and/or regulatory element can result in alteration or modification of the expression of each gene product encoded by the operon. Alteration or modification of the regulatory element can include, but is not limited to removing the endogenous promoter and/or regulatory element(s), adding strong promoters, inducible promoters or multiple promoters or removing regulatory sequences such that expression of the gene products is modified, modifying the chromosomal location of the operon, altering nucleic acid sequences adjacent to the operon or within the operon such as a ribosome binding site, increasing the copy number of the operon, modifying proteins (*e.g.*, regulatory proteins, suppressors, enhancers, transcriptional activators and the like) involved in transcription of the operon and/or translation of the gene products of the operon, or any other conventional means of deregulating expression of genes routine in the art (including but not limited to use of antisense nucleic acid molecules, for example, to block expression of repressor proteins). Deregulation can also involve altering the coding region of one or more genes to yield, for example, an enzyme that is feedback resistant or has a higher or lower specific activity.

In another preferred embodiment, a recombinant microorganism is designed or engineered such that at least one pantothenate biosynthetic enzyme, at least one isoleucine-valine biosynthetic enzyme, and/or at least one HMBPA biosynthetic enzyme is overexpressed. The term "overexpressed" or "overexpression" includes expression of a gene product (*e.g.*, a biosynthetic enzyme) at a level greater than that expressed prior to manipulation of the microorganism or in a comparable microorganism which has not been manipulated. In one embodiment, the microorganism can be genetically designed or engineered to overexpress a level of gene product greater than that expressed in a comparable microorganism which has not been engineered.

Genetic engineering can include, but is not limited to, altering or modifying regulatory sequences or sites associated with expression of a particular gene (*e.g.*, by adding strong promoters, inducible promoters or multiple promoters or by removing regulatory sequences such that expression is constitutive), modifying the chromosomal location of a particular gene, altering nucleic acid sequences adjacent to a particular gene such as a ribosome binding site, increasing the copy number of a particular gene, modifying proteins (*e.g.*, regulatory proteins, suppressors, enhancers, transcriptional activators and the like) involved in transcription of a particular gene and/or translation of a particular gene product, or any other conventional means of deregulating expression of a particular gene routine in the art (including but not limited to use of antisense nucleic acid molecules, for example, to block expression of repressor proteins). Genetic engineering can also include deletion of a gene, for example, to block a pathway or to remove a repressor.

In another embodiment, the microorganism can be physically or environmentally manipulated to overexpress a level of gene product greater than that expressed prior to manipulation of the microorganism or in a comparable microorganism which has not been manipulated. For example, a microorganism can be treated with or cultured in the presence of an agent known or suspected to increase transcription of a particular gene and/or translation of a particular gene product such that transcription and/or translation are enhanced or increased. Alternatively, a microorganism can be cultured at a temperature selected to increase transcription of a particular gene and/or translation of a particular gene product such that transcription and/or translation are enhanced or increased.

V. Culturing and Fermenting Recombinant Microorganisms

The term "culturing" includes maintaining and/or growing a living microorganism of the present invention (*e.g.*, maintaining and/or growing a culture or strain). In one embodiment, a microorganism of the invention is cultured in liquid media. In another embodiment, a microorganism of the invention is cultured in solid media or semi-solid media. In a preferred embodiment, a microorganism of the invention is cultured in media (*e.g.*, a sterile, liquid medium) comprising nutrients essential or beneficial to the maintenance and/or growth of the microorganism (*e.g.*, carbon sources or carbon substrate, for example carbohydrate, hydrocarbons, oils, fats, fatty acids, organic acids, and alcohols; nitrogen sources, for example, peptone, yeast extracts, meat extracts, malt extracts, urea, ammonium sulfate, ammonium chloride, ammonium nitrate and ammonium phosphate; phosphorus sources, for example, phosphoric acid, sodium and potassium salts thereof; trace elements, for example, magnesium, iron, manganese, calcium, copper, zinc, boron, molybdenum, and/or cobalt salts; as well as growth factors such as amino acids, vitamins, growth promoters and the like).

Preferably, microorganisms of the present invention are cultured under controlled pH. The term "controlled pH" includes any pH which results in production of the desired product (*e.g.*, pantoate and/or pantothenate). In one embodiment microorganisms are cultured at a pH of about 7. In another embodiment, microorganisms are cultured at a pH of between 6.0 and 8.5. The desired pH may be maintained by any number of methods known to those skilled in the art.

Also preferably, microorganisms of the present invention are cultured under controlled aeration. The term "controlled aeration" includes sufficient aeration (*e.g.*, oxygen) to result in production of the desired product (*e.g.*, pantoate and/or pantothenate). In one embodiment, aeration is controlled by regulating oxygen levels in the culture, for example, by regulating the amount of oxygen dissolved in culture media. Preferably, aeration of the culture is controlled by agitating the culture. Agitation may be provided by a propeller or similar mechanical agitation equipment, by revolving or shaking the cuture vessel (*e.g.*, tube or flask) or by various pumping equipment. Aeration may be further controlled by the passage of sterile air or oxygen through the medium (*e.g.*, through the fermentation mixture). Also preferably, microorganisms of the present invention are cultured without excess foaming (*e.g.*, *via* addition of antifoaming agents).

Moreover, microorganisms of the present invention can be cultured under controlled temperatures. The term "controlled temperature" includes any temperature

which results in production of the desired product (*e.g.*, pantoate and/or pantothenate). In one embodiment, controlled temperatures include temperatures between 15°C and 95°C. In another embodiment, controlled temperatures include temperatures between 15°C and 70°C. Preferred temperatures are between 20°C and 55°C, more preferably between 30°C and 50°C.

Microorganisms can be cultured (*e.g.*, maintained and/or grown) in liquid media and preferably are cultured, either continuously or intermittently, by conventional culturing methods such as standing culture, test tube culture, shaking culture (*e.g.*, rotary shaking culture, shake flask culture, etc.), aeration spinner culture, or fermentation. In a preferred embodiment, the microorganisms are cultured in shake flasks. In a more preferred embodiment, the microorganisms are cultured in a fermentor (*e.g.*, a fermentation process). Fermentation processes of the present invention include, but are not limited to, batch, fed-batch and continuous processes or methods of fermentation. The phrase "batch process" or "batch fermentation" refers to a system in which the composition of media, nutrients, supplemental additives and the like is set at the beginning of the fermentation and not subject to alteration during the fermentation, however, attempts may be made to control such factors as pH and oxygen concentration to prevent excess media acidification and/or microorganism death. The phrase "fed-batch process" or "fed-batch" fermentation refers to a batch fermentation with the exception that one or more substrates or supplements are added (*e.g.*, added in increments or continuously) as the fermentation progresses. The phrase "continuous process" or "continuous fermentation" refers to a system in which a defined fermentation media is added continuously to a fermentor and an equal amount of used or "conditioned" media is simultaneously removed, preferably for recovery of the desired product (*e.g.*, pantoate and/or pantothenate). A variety of such processes have been developed and are well-known in the art.

The phrase "culturing under conditions such that a desired compound is produced" includes maintaining and/or growing microorganisms under conditions (*e.g.*, temperature, pressure, pH, duration, etc.) appropriate or sufficient to obtain production of the desired compound or to obtain desired yields of the particular compound being produced. For example, culturing is continued for a time sufficient to produce the desired amount of a compound (*e.g.*, pantoate and/or pantothenate). Preferably, culturing is continued for a time sufficient to substantially reach suitable production of the compound (*e.g.*, a time sufficient to reach a suitable concentration of pantoate and/or pantothenate or suitable ratio of pantoate and/or pantothenate:HMBPA). In one embodiment, culturing is continued for about 12 to 24 hours. In another embodiment,

culturing is continued for about 24 to 36 hours, 36 to 48 hours, 48 to 72 hours, 72 to 96 hours, 96 to 120 hours, 120 to 144 hours, or greater than 144 hours. In yet another embodiment, microorganisms are cultured under conditions such that at least about 5 to 10 g/L of compound are produced in about 36 hours, at least about 10 to 20 g/L compound are produced in about 48 hours, or at least about 20 to 30 g/L compound in about 72 hours. In yet another embodiment, microorganisms are cultured under conditions such that at least about 5 to 20 g/L of compound are produced in about 36 hours, at least about 20 to 30 g/L compound are produced in about 48 hours, or at least about 30 to 50 or 60 g/L compound in about 72 hours. In another embodiment, microorganisms are cultured under conditions such that a ratio of HMBPA:HMBPA+pantothenate of 0.1:100 or less is achieved (*i.e.*, 0.1% HMBPA versus 99.9% pantothenate, for example, as determined by comparing the peak areas when a sample of product is analyzed by HPLC), preferably such that a ratio of 0.2:100 or less is achieved (0.2% HMBPA versus 99.8% pantotheante), more preferably such that a ratio of 0.5:100 or less is achieved (0.5% HMBPA versus 99.5% pantotheante). In yet another embodiment, microorganisms are cultured under conditions such that a ratio of HMBPA:HMBPA+pantothenate of 1:100 or less is achieved (*i.e.*, 1% HMBPA versus 99% pantothenate, for example, as determined by comparing the peak areas when a sample of product is analyzed be HPLC), preferably such that a ratio of 2:100 or less is achieved (2% HMBPA versus 98% pantotheante), more preferably such that a ratio of 3:100 or less is achieved (3% HMBPA versus 97% pantotheante), more preferably at least 4:100 or less (4% HMBPA versus 96% pantotheante), 5:100 or less (5% HMBPA versus 95% pantotheante), 6:100 or less (6% HMBPA versus 94% pantotheante), 7:100 or less (7% HMBPA versus 93% pantotheante), 8:100 or less (8% HMBPA versus 92% pantotheante), 9:100 or less (9% HMBPA versus 91% pantotheante), or 10:100 or less (10% HMBPA versus 90% pantotheante).

The methodology of the present invention can further include a step of recovering a desired compound (*e.g.*, pantoate and.or pantothenate). The term "recovering" a desired compound includes extracting, harvesting, isolating or purifying the compound from culture media. Recovering the compound can be performed according to any conventional isolation or purification methodology known in the art including, but not limited to, treatment with a conventional resin (*e.g.*, anion or cation exchange resin, non-ionic adsorption resin, etc.), treatment with a conventional adsorbent (*e.g.*, activated charcoal, silicic acid, silica gel, cellulose, alumina, etc.), alteration of pH, solvent extraction (*e.g.*, with a conventional solvent such as an alcohol, ethyl acetate, hexane and the like), dialysis, filtration, concentration, crystallization,

recrystallization, pH adjustment, lyophilization and the like. For example, a compound can be recovered from culture media by first removing the microorganisms from the culture. Media are then passed through or over a cation exchange resin to remove cations and then through or over an anion exchange resin to remove inorganic anions and organic acids having stronger acidities than the compound of interest. The resulting compound can subsequently be converted to a salt (*e.g.*, a calcium salt) as described herein.

Preferably, a desired compound of the present invention is "extracted", "isolated" or "purified" such that the resulting preparation is substantially free of other media components (*e.g.*, free of media components and/or fermentation byproducts). The language "substantially free of other media components" includes preparations of the desired compound in which the compound is separated from media components or fermentation byproducts of the culture from which it is produced. In one embodiment, the preparation has greater than about 80% (by dry weight) of the desired compound (*e.g.*, less than about 20% of other media components or fermentation byproducts), more preferably greater than about 90% of the desired compound (*e.g.*, less than about 10% of other media components or fermentation byproducts), still more preferably greater than about 95% of the desired compound (*e.g.*, less than about 5% of other media components or fermentation byproducts), and most preferably greater than about 98-99% desired compound (*e.g.*, less than about 1-2% other media components or fermentation byproducts). When the desired compound has been derivatized to a salt, the compound is preferably further free of chemical contaminants associated with the formation of the salt. When the desired compound has been derivatized to an alcohol, the compound is preferably further free of chemical contaminants associated with the formation of the alcohol.

In an alternative embodiment, the desired compound is not purified from the microorganism, for example, when the microorganism is biologically non-hazardous (*e.g.*, safe). For example, the entire culture (or culture supernatant) can be used as a source of product (*e.g.*, crude product). In one embodiment, the culture (or culture supernatant) is used without modification. In another embodiment, the culture (or culture supernatant) is concentrated. In yet another embodiment, the culture (or culture supernatant) is dried or lyophilized.

Preferably, a production method of the present invention results in production of the desired compound at a significantly high yield. The phrase "significantly high yield" includes a level of production or yield which is sufficiently elevated or above what is usual for comparable production methods, for example, which

is elevated to a level sufficient for commercial production of the desired product (*e.g.*, production of the product at a commercially feasible cost). In one embodiment, the invention features a production method that includes culturing a recombinant microorganism under conditions such that the desired product (*e.g.*, pantoate and/or pantothenate) is produced at a level greater than 2 g/L. In another embodiment, the invention features a production method that includes culturing a recombinant microorganism under conditions such that the desired product (*e.g.*, pantoate and/or pantothenate) is produced at a level greater than 10 g/L. In another embodiment, the invention features a production method that includes culturing a recombinant microorganism under conditions such that the desired product (*e.g.*, pantoate and/or pantothenate) is produced at a level greater than 20 g/L. In yet another embodiment, the invention features a production method that includes culturing a recombinant microorganism under conditions such that the desired product (*e.g.*, pantoate and/or pantothenate) is produced at a level greater than 30 g/L. In yet another embodiment, the invention features a production method that includes culturing a recombinant microorganism under conditions such that the desired product (*e.g.*, pantoate and/or pantothenate) is produced at a level greater than 40 g/L. In yet another embodiment, the invention features a production method that includes culturing a recombinant microorganism under conditions such that the desired product (*e.g.*, pantoate and/or pantothenate) is produced at a level greater than 50 g/L. In yet another embodiment, the invention features a production method that includes culturing a recombinant microorganism under conditions such that the desired product (*e.g.*, pantoate and/or pantothenate) is produced at a level greater than 60 g/L. The invention further features a production method for producing the desired compound that involves culturing a recombinant microorganism under conditions such that a sufficiently elevated level of compound is produced within a commercially desirable period of time.

Depending on the biosynthetic enzyme or combination of biosynthetic enzymes manipulated, it may be desirable or necessary to provide (*e.g.*, feed) microorganisms of the present invention at least one biosynthetic precursor such that the desired compound or compounds are produced. The term "biosynthetic precursor" or "precursor" includes an agent or compound which, when provided to, brought into contact with, or included in the culture medium of a microorganism, serves to enhance or increase biosynthesis of the desired product. In one embodiment, the biosynthetic precursor or precursor is aspartate. In another embodiment, the biosynthetic precursor or precursor is β-alanine. The amount of aspartate or β-alanine added is preferably an amount that results in a concentration in the culture medium sufficient to enhance

productivity of the microorganism (*e.g.*, a concentration sufficient to enhance production of pantoate and/or pantothenate). Biosynthetic precursors of the present invention can be added in the form of a concentrated solution or suspension (*e.g.*, in a suitable solvent such as water or buffer) or in the form of a solid (*e.g.*, in the form of a powder). Moreover, biosynthetic precursors of the present invention can be added as a single aliquot, continuously or intermittently over a given period of time. The term "excess β-alanine" includes β-alanine levels increased or higher that those routinely utilized for culturing the microorganism in question. For example, culturing the *Bacillus* microorganisms described in the instant Examples is routinely done in the presence of about 0-0.01 g/L β-alanine. Accordingly, excess β-alanine levels can include levels of about 0.01-1, preferably about 1-20 g/L.

In yet another embodiment, the biosynthetic precursor is valine. In yet another embodiment, the biosynthetic precursor is α-ketoisovalerate. Preferably, valine or α-ketoisovalerate is added in an amount that results in a concentration in the medium sufficient for production of the desired product (*e.g.*, pantoate and/or pantothenate) to occur. The term "excess α-KIV" includes α-KIV levels increased or higher that those routinely utilized for culturing the microorganism in question. For example, culturing the *Bacillus* microorganisms described in the instant Examples is routinely done in the presence of about 0-0.01 g/L α-KIV. Accordingly, excess α-KIV levels can include levels of about 0.01-1, preferably about 1-20 g/L α-KIV. The term "excess valine" includes valine levels increased or higher that those routinely utilized for culturing the microorganism in question. For example, culturing the *Bacillus* microorganisms described in the instant Examples is routinely done in the presence of about 0-0.5 g/L valine. Accordingly, excess valine levels can include levels of about 0.5-5 g/L, preferably about 5-20 g/L valine. Biosynthetic precursors are also referred to herein as "supplemental biosynthetic substrates".

Another aspect of the present invention includes biotransformation processes which feature the recombinant microorganisms described herein. The term "biotransformation process", also referred to herein as "bioconversion processes", includes biological processes which results in the production (*e.g.*, transformation or conversion) of appropriate substrates and/or intermediate compounds into a desired product (*e.g.*, pantoate and/or pantothenate).

The microorganism(s) and/or enzymes used in the biotransformation reactions are in a form allowing them to perform their intended function (*e.g.*, producing a desired compound). The microorganisms can be whole cells, or can be only those portions of the cells necessary to obtain the desired end result. The microorganisms can

be suspended (*e.g.*, in an appropriate solution such as buffered solutions or media), rinsed (*e.g.*, rinsed free of media from culturing the microorganism), acetone-dried, immobilized (*e.g.*, with polyacrylamide gel or k-carrageenan or on synthetic supports, for example, beads, matrices and the like), fixed, cross-linked or permeablized (*e.g.*, have permeablized membranes and/or walls such that compounds, for example, substrates, intermediates or products can more easily pass through said membrane or wall).

VI. Processes for the Production of Selectively Mixed Compositions of Pantothenate and HMBPA

The present invention further features processes and microorganisms for the production of selectively mixed compositions of pantothenate and HMBPA. As defined herein, the phrase "selectively mixed composition" includes a composition produced in a manner such that the ratio of pantothenate to HMBPA is a controlled feature, *i.e.*, the ratio of pantothenate to HMBPA is selected. The selection can occur by manipulating the microorganism producing the composition (*i.e.*, the production strain) such that one component is favored for production over the other.

In one aspect, the invention features a process for the production of a selectively mixed pantothenate:HMBPA composition that includes culturing a microorganism having a deregulated pantothenate biosynthetic pathway under conditions such that a selectively mixed pantothenate:HMBPA composition is produced. In one embodiment, the microorganism is cultured under conditions that favor pantothenate production. In another embodiment, the microorganism is cultured under conditions that favor HMBPA production. In another embodiment, the microorganism is cultured under conditions of controlled steady state glucose that favor pantothenate production. In another embodiment, the microorganism is cultured under conditions of controlled steady state glucose that favor HMBPA production. In yet another embodiment, the microorganism is cultured under conditions of controlled steady state dissolved oxygen that favor pantothenate production. In yet another embodiment, the microorganism is cultured under conditions of controlled steady state dissolved oxygen that favor HMBPA production. In yet another embodiment, the microorganism is cultured under conditions of controlled serine levels that favor pantothenate production. In one embodiment, the composition comprises pantothenate and HMBPA at a ratio of 75 mol pantothenate to 25 mol HMBPA or greater. In another embodiment, the composition comprises pantothenate and HMBPA at a ratio of 90 mol pantothenate to 10 mol HMBPA or greater. In another embodiment, the composition comprises

pantothenate and HMBPA at a ratio of 75 mol HMBPA to 25 mol pantothenate or greater. In yet another embodiment, the composition comprises pantothenate and HMBPA at a ratio of 90 mol HMBPA to 10 mol pantothenate or greater. Values and ranges included and/or intermediate of the values set forth herein are also intended to be within the scope of the present invention.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are incorporated herein by reference.

EXAMPLES

Example I: Discovery and Characterization of the [R]-3-(2-hydroxy-3-methyl-butyrylamino)-propionic acid (HMBPA) Biosynthetic Pathway

In developing *Bacillus* strains for the production of pantothenate, various genetic manipulations are made to genes and enzymes involved in the pantothenate biosynthetic pathway and the isoleucine-valine (*ilv*) pathway (Figure 1) as described in U.S. Patent Application Serial No. 09/400,494 and U.S. Patent Application Serial No. 09/667,569. For example, strains having a deregulated *panBCD* operon and/or having deregulated *panE1* exhibit enhanced pantothenate production (when cultured in the presence of β-alanine and α-ketoisovalerate (α-KIV)). Strains further deregulated for *ilvBNC* and *ilvD* exhibit enhanced pantothenate production in the presence of only β-alanine. Moreover, it is possible to achieve β-alanine independence by further deregulating *panD*.

An exemplary strain is PA824, a tryptophan prototroph, Spec and Tet resistant, deregulated for *panBCD* at the *panBCD* locus, deregulated for *panE1* at the *panE1* locus (two genes in the *B. subtilis* genome are homologous to *E. coli panE*, *panE1* and *panE2*, the former encoding the major ketopantoate reductase involved in pantothenate production, while *panE2* does not contribute to pantothenate synthesis (U.S. Patent Application Serial No. 09/400,494), deregulated for *ilvD* at the *ilvD* locus, overexpressing an *ilvBNC* cassette at the *amyE* locus, and overexpressing *panD* at the *bpr* locus.

Under the following fermentation conditions, PA824 routinely yields approximately 20-30 g/L pantothenate. The production of pantothenate by PA824 in this example is accomplished in 14 L fermentor vessels. The composition of the batch and feed media are as follows.

BATCH

	MATERIAL	g/L (final)
1	Yeast extract	10
2	Na Glutamate	5
3	$(NH_4)_2SO_4$	8
4	KH_2PO_4	5
5	K_2HPO_4	7.6

Added After Sterilization and Cool Down

1	Glucose	2.5
2	$CaCl_2$	0.1
3	$MgCl_2$	1
4	Sodium Citrate	1
5	$FeSO_4 \cdot 7\ H_2O$	0.01
5	SM-1000X	1 ml

The final volume of the batch medium is 6 L. The trace element solution SM-1000X has following composition: 0.15 g $Na_2MoO_4 \cdot 2\ H_2O$, 2.5 g H_3BO_3, 0.7 g $CoCl_2 \cdot 6\ H_2O$, 0.25 g $CuSO_4 \cdot 5\ H_2O$, 1.6 g $MnCl_2 \cdot 4\ H_2O$, 0.3 g $ZnSO_4 \cdot 7\ H_2O$ are dissolved in water (final volume 1L).

The batch medium was inoculated with 60 ml of shake flask PA824 culture (OD=10 in SVY medium: Difco Veal Infusion broth 25 g, Difco Yeast extract 5 g, Sodium Glutamate 5 g, $(NH_4)_2SO_4$ 2.7 g in 740 ml H_2O, autoclave; add 200 ml sterile 1 M K_2HPO_4 (pH 7) and 60 ml sterile 50% Glucose solution (final volume 1L)). The fermentation was run at 43 °C at an air flow rate of 12 L/min as a glucose limited fed batch. The initial batched glucose (2.5 g/L) was consumed during exponential growth. Afterwards glucose concentrations were maintained between 0.2-1 g/L by continuous feeding of FEED solution as follows.

FEED

	MATERIAL	**g/L (final)**
1	Glucose	550
2	$CaCl_2$	0.1
3	SM-1000X	3 ml

The variable feed rate pump was computer controlled and linked to the glucose concentration in the tank by an algorithm. In this example the total feeding was 6 L.

During fermentation the pH was set at 7.2. Control was achieved by pH measurements linked to computer control. The pH value was maintained by feeding either a 25% NH_3-solution or a 20% H_3PO_4-solution. NH_3 acts simultaneously as a N-

source for the fermentation. The dissolved oxygen concentration [pO_2] was set at 30% by regulation of the agitation and aeration rate. Foaming was controlled by addition of silicone oil. After the stop of the addition of the feed solution, in this example after 48 hours, the fermentation was continued until the [pO_2] value reached 95%. Then the fermentation was stopped by killing the microorganism through sterilization for 30 min. The successful sterilization was proven by plating a sample of the fermentation broth on agar plates. The pantothenate titer in the fermentation broth was 21.7 g/L after sterilization and removal of the cells by centrifugation (determined by HPLC analysis).

For HPLC analysis the fermentation broth sample was diluted with sterile water (1:40). 5 µl of this dilution was injected into a HPLC column (Aqua C18, 5µm, 150x2.0 mm, Phenomenex™). Temperature of the column was held at 40°C. Mobile phase A was 14.8 mM H_3PO_3, mobile phase B 100% Acetonitrile. Flow rate was constant at 0.5 mL/min. A gradient was applied :

start:	2% mobile phase B
0-3 min	linear increase to 3% mobile phase B
3-3.5 min	linear increase to 20% mobile phase B

The detection was carried out by an UV-detector (210 nm). Run time was 7 min with an additional 3 min posttime. The retention time for pantothenic acid is 3.9 minutes. The HPLC chromatogram for the above mentioned sample is given in Figure 4.

Identification of a compound related to the peak with retention time 4.7 minutes

In addition to producing significant quantities of pantothenate, it was discovered a second compound eluted with an approximate retention time of 4.7 minutes in this system. The second prominent product formed in the fermentation was shown to be 3-(2-hydroxy-3-methyl-butyrylamino)-propionic acid (HMBPA) (also referred to herein as "β-alanine 2-(*R*)-hydroxyisolvalerate", "β-alanine 2-hydroxyisolvalerate", "β-alanyl-α-hydroxyisovalarate" and/or "fantothenate"). It was identified by its mass spectrum (Figure 5; relative monoisotopic mass 189), and by ^{1}H- and 13C-NMR after chromatographic purification by reverse phase flash chromatography (mobile phase 10 mM KH_2PO_4, with increasing contents of acetonitrile (1-50%)) (data not shown). The compound was presumed to have the R configuration at the assymetric carbon by analogy with [R]-pantothenate.

In order to verify the identity of the compound, deliberate synthesis of racemic β-alanine 2-hydroxyisolvalerate was performed as follows. β-alanine (2.73 g / 30 mmol) and sodium methoxide (5.67 g of a 30% solution in methanol / 31.5 mmol) were dissolved in methanol (40 mL). Methyl 2-hydroxyisovalerate (2-hydroxy-3-

methylbutyric acid methyl ester) (3.96g / 30 mmol) was added and refluxed for 18 hours. Methanol was then removed by rotavap and replaced by tert-butanol (50 mL). Potassium tert-butoxide was added (50 mg) and refluxed for 26 hours. The solvent was removed *in vacuo*, the residue dissolved in water (50 mL) and passed through a strongly acidic ion-exchange resin (H+-form Lewatite™ S 100 G1; 100 mL). More water is used to rinse the ion exchanger. The aqueous eluates are combined and the water removed *in vacuo*. The residue is subjected to flash chromatography (silica gel; 2% acetic acid in ethyl acetate as eluent) and the product fractions evaporated to give a solid residue. The residue was recrystallized from ethyl acetate / toluene (10 mL / 20 mL, respectively) and analytically pure HMBPA (β-alanine 2-hydroxyisolvalerate) was obtained, which showed a relative monoisotopic mass of 190 (189 + H^+) in the mass spectrometer and the same ^{1}H-NMR resonances as the product obtained from fermentation.

The biosynthetic pathway resulting in HMBPA production is set forth in Figure 2. The chemical structure of [R]-3-(2-hydroxy-3-methyl-butyrylamino)-propionic acid (HMBPA) is depicted in Figure 3. As depicted in Figure 2, HMBPA is the condensation product of [R]-α-hydroxyisovaleric acid (α-HIV) and β-alanine, catalyzed by the PanC enzyme. α-HIV is generated by reduction of α-KIV, a reaction that is catalyzed by the α-keto reductases PanE (*e.g.*, PanE1 and/or PanE2) and/or IlvC.

Based on the chemical structure and biosynthetic pathway leading to HMBPA production, the present inventors have formulated the following model to describe the interaction between the previously known pantothenate and isoleucine-valine (*ilv*) pathways and the newly characterized HMBPA biosynthetic pathway. In at least one aspect, the model states that there exist at least two pathways in microorganisms that compete for α-KIV, the substrate for the biosynthetic enzyme PanB, namely the pantothenate biosynthetic pathway and the HMBPA biosynthetic pathway. (A third and fourth pathway competing for α-KIV are those resulting in the production of valine or leucine from α-KIV, see *e.g.*, Figure 1). At least the pantothenate biosynthetic pathway and the HMBPA biosynthetic pathway further produce competitive substrates for the enzyme PanC, namely α-HIV and pantoate. Production of HMBPA has significant effects on pantothenate production. Most importantly, the HMBPA pathway competes with the pantothenate pathway for precursors (α-KIV and β-alanine) and for some of the enzymes (PanC, PanD, PanE1, and/or IlvC). In addition, because the structure of HMBPA is similar to that of pantothenate, it may have the undesirable property of negatively regulating one or more steps in the pantothenate pathway. The model predicts that production of pantothenate can be improved or optimized by any means which favor use of substrates (α-KIV and

β-alanine) and/or enzymes (PanC, PanD, PanE1, and/or IlvC) in pantothenate biosynthetic processes as compared to HMBPA biosynthetic processes.

A preferred approach to maximize pantoate and/or pantothenate production while minimizing HMBPA production is to increase the activity of PanB in the cells because this will decrease the availability of α-KIV for α-HIV synthesis while promoting the synthesis of ketopantoate and pantoate. Methods of increasing activity include overexpressing the *panB* gene, increasing the copy number of the gene, increasing the specific activity of the enzyme and/or relieving inhibition of the enzyme by mutation or by lowering Coenzyme A levels. Higher pantoate levels in turn increase pantothenate synthesis and decrease HMBPA synthesis by out competing α-HIV for PanC.

Another approach to maximize pantothenate synthesis is to optimize the level of *panE1* expression. It is demonstrated herein that increasing production of PanE1 increases the synthesis of HMBPA at the expense of pantothenate. Accordingly, effecting a moderate decrease in the level of *panE1* expression in PA824 or PA668 (*i.e.*, precisely regulating the level of *panE1* expression) will result in a decrease in HMBPA synthesis and an increase in pantothenate synthesis. Moreover, as shown in Example II, deletion of *panE2* significantly decreases HMBPA synthesis.

Other approaches to increasing pantoate and/or pantothenate synthesis versus HMBPA synthesis include optimizing α-KIV production levels in the cells and/or isolating a modified PanC protein with increased preference for pantoate as a substrate.

The following examples provide experimental support for the model described herein and further exemplify processes for increasing the production of pantoate and/or pantothenate (relative to HMBPA levels) based on the model.

EXAMPLES II-VIII:

For Examples II-VI, quantitation of pantothenate and/or HMBPA was performed as follows. Aliquots of fermentation media were diluted 1:100 and aliquots of test tube cultures were diluted 1:10 in water or 5% acetonitrile prior to injection on a Phenomenex Aqua™ 5μ C18 HPLC column (250 x 4.60mm, 125A). Mobile phases were A = 5% acetonitrile, 50 mM monosodium phosphate buffer adjusted to pH 2.5 with phosphoric acid; and B = 95% acetonitrile, 5% H_2O.

Linear gradients were as follows.

Minutes	Solvent A	Solvent B
0	100%	0%
16	100%	0%
17	0%	100%
20	0%	100%
21	100%	0%

Additional parameters and apparatus were as follows: Flow rate = 1.0 ml/min; Injection volume = 20 µl; Detector = Hewlett Packard 1090 series DAD UV detector-3014, Signal A = 197 nm, ref. = 450 nm, Firmware revision E; Column heater = Oven temperature 40°C; Hardware = Hewlett Packard Kayak™ XA; and Software = Hewlett Packard Chemstation Plus™ family revision A.06.03[509].

HMBPA elutes at approximately 13 minutes in this system.

Example II: Decreasing HMBPA Synthesis by Deleting PanE2 From Pantothenate Production Strains

As described in Example I, HMBPA production was first observed in microorganisms overexpressing *panE1* indicating that ketopantoate reductase is capable of catalyzing not only the reduction of ketopantoate to pantoate but also the reduction of α-ketoisovalerate to 2-hydroxyisovalerate. As mentioned previously, two genes in the *B. subtilis* genome are homologous to the *E. coli panE* gene encoding ketopantoate reductase and have been named *panE1* and *panE2*. In *Bacillus*, it has been demonstrated that the *panE1* gene encodes the major ketopantoate reductase involved in pantothenate production, whereas *panE2* does not contribute to pantothenate synthesis. Moreover, overexpression of *panE2* from a P_{26}*panE2* cassette in pAN238 (SEQ ID NO:25) leads to a reduction in pantothenate titer (see *e.g.*, U.S. Patent Application Serial No. 09/400,494). Given the homology between the *panE2* and *panE1* gene products and the fact that overexpression of *panE2* shifted production away from pantoate/pantothenate, it was tested whether *panE2* contributed in any significant manner to the production of HMBPA. It was hypothesized that the *panE2* gene product is an enzyme capable of

reducing α-KIV to α-HIV, but incapable of significantly reducing ketopantoate to pantoate.

To test the hypothesis, *panE2* was deleted from pantothenate production strain PA824 (described in Example I) by transforming with a *ΔpanE2::cat* cassette from chromosomal DNA of strain PA248 (*ΔpanE2::cat*) (set forth as SEQ ID NO:24, for construction see *e.g.*, U.S. Patent Application Serial No. 09/400,494) to give strain PA919. Three isolates of PA919 were compared to PA824 for pantothenate and HMBPA production in test tube cultures grown in SVY plus β-alanine.

Table 1. Production of pantothenate and HMBPA by derivatives of PA824 and PA880 grown at 43^{0}C in 48 hour test tube cultures of SVY glucose + β-alanine[5].

Strain	new trait	parent	OD_{600}	[pan] g/l	[HMBPA] g/l
PA824	_		13.9	4.3	0.64
PA880	Δ*coaX*	PA824	16.4	5.1	0.48
PA894	Δ*coaX, coaA*(S151L), *cat*	PA880	14.9	4.9	0.47
PA911-5	P_{26}*panB@ vpr,* Δ*coaX*	PA880	13.4	5.3	0.40
PA911-8	P_{26}*panB@ panB,* Δ*coaX*	"	13.8	6.1	0.45
PA919-1	Δ*panE2::cat*	PA824	13.2	4.2	0.15
PA919-2	"	"	14.8	3.8	0.13
PA919-3	"	"	18.0	5.5	0.14

As indicated by the data in Table 1, all three isolates of PA919 produced about four-fold lower HMBPA than PA824 demonstrating that the *panE2* gene product contributed to HMBPA production and demonstrating that HMBPA production can be at least partially eliminated by simply deleting *panE2*, without sacrificing pantothenate production.

Example III. HMBPA Production and Pantothenate Production are Inversely Correlated.

Strains derived from PA365 (the RL-1 lineage equivalent of PA377, described in U.S. patent application Serial No. 09/667,569) which are deleted for the *P26 panBCD* cassette and which contain a *P26 panC*D* cassette amplified at the *vpr* locus and either the wild type *P26 panB* cassette (PA666) or a *P26 ΔpanB* cassette (PA664) amplified at the *bpr* locus were constructed as follows. An alignment of the C-terminal amino acids of known or suspected PanB proteins is shown in Figure 6. Three regions called 1, 2 and 3 were identified having conserved or semi-conserved amino acid residues that are indicated by arrows at the top of the figure. The *B. subtilis* PanB protein (RBS02239) is underlined. Two of the PanB proteins (RCY14036 and CAB56202.1) are missing region 3 while the latter PanB protein is also missing region 2 and has non-conserved amino acid residues occupying region 1.

B. subtilis PanB variants were created that were missing regions 1, 2 and 3. The desired variants were created by designing 3' PCR primers to amplify the *B. subtilis pan B* gene such that region 3, regions 2 and 3, or all three regions would be missing from the final product. The PCR products were generated and cloned into *E. coli* expression vector pASK-1BA3, creating plasmids pAN446, pAN447, and pAN448, respectively. The plasmids were then transformed into *E. coli* strain SJ2 that contains the *panB6* mutation to test for complementation. Only pAN446, which is missing region 3, was able to complement. This indicates that region 3 is not essential for *B. subtilis* PanB activity but that region 2 is required for activity or stability.

The next step in this analysis was to transfer the *panB* gene from pAN446 to a *B. subtilis* expression vector and then introduce it into a strain appropriate for testing activity of the encoded PanB protein in *B. subtilis*. To do this, a strain that is deleted for the *P26 panBCD* operon was first created. This was accomplished by first inserting a *cat* gene between the *Bse*RI site located just upstream of the *panB* RBS and the *Bg*/II site located in *pan*D, creating plasmid pAN624, SEQ ID NO:20 (*Figure 7*).

The resulting deletion-substitution mutation (Δ*panB*CD::*cat*624), which removes all of *panB* and *panC*, was crossed into PA354 by transformation, with selection for resistance to chloramphenicol on plates supplemented with 1 mM pantothenate. One of the transformants was saved and named PA644. Chromosomal DNA isolated from PA644 was analyzed by PCR and was shown to contain the deletion-substitution mutation. As expected, PA644 requires pantothenate for growth but retains the engineered *ilv* genes (P_{26}*ilvBNC* P_{26}*ilvD*) as well as the P_{26}*pan E1* gene originally present in PA354. Thus, it has all the enzymes involved in pantoate synthesis overproduced except PanB. The gene containing the shortest *panB* deletion was inserted into *B. subtilis* expression vector pOTP61 (described in US patent application Serial No. 09/667,569), creating plasmid pAN627. At the same time, a wild-type *panB* control gene was inserted into pOTP61, creating plasmid pAN630. The *Not*I fragments of each plasmid, lacking *E. coli* vector sequences, were ligated and transformed into PA644, with selection for resistance to tetracycline.

One transformant from each transformation was saved and further transformed with chromosomal DNA from PA628 with selection for Pan^+. PA628 contains a multicopy P_{26}*panC*D* expression plasmid (*pAN620*) integrated at the *vpr* locus. In order to determine the effects of the *panB* gene mutation directly on pantothenate production, plasmid pAN620 SEQ ID NO:21, which is illustrated in *Figure 8*, provides the remaining two enzymes required for pantothenate synthesis (PanC and PanD). Four transformants from each transformation were isolated, grown in SVY medium containing 10 g/L aspartate for 48 hours, then assayed for pantothenate production. Transformants with the 3' deleted *panB* gene were named PA664 and those containing the wild-type gene were called PA666. The data showed that the 3' deleted *panB* gene in PA664 encodes a PanB protein with greatly reduced activity. To test for HMBPA production, test tube cultures of PA365, PA666, and PA664 were grown in SVY + aspartate medium with and without added α-KIV or pantoate for 48 hours and then assayed for HMBPA and pantothenate as described previously.

Table 2. Effect of PanB activity and addition of precursors on HMBPA and pantothenate production, 48 hour test tube culture data, SVY + aspartate (10 g/L) medium.

				no additions		+ α-KIV (5 g/L)		+ pantoate (5 g/L)	
Strain	**pan operon**	**panC*D plasmid**	**panB plasmid**	**[pan] (g/L)**	**HMBPA peak***	**[pan] (g/L)**	**HMBPA peak**	**[pan] (g/L)**	**HMBPA peak**
PA365	*P_{26} panBCD*	NONE	NONE	3.0	0.71	3.2	1.28	4.8	0.38
PA666	*ΔpanBCD::cat*	pAN620	pAN630	3.7	0.55	3.3	1.70	5.2	0.26
PA664	*ΔpanBCD::cat*	pAN620	pAN627	0.3	1.39	0.6	1.76	2.5	0.74

* HMBPA peak = peak area X 10^{-3}

The data presented in Table 2 demonstrate that in the absence of supplements, PA666 produced the least HMBPA whereas PA664 produced the most, indicating an inverse correlation between PanB activity and HMBPA production. This is consistent with the model which predicts that the two pathways compete for α-KIV, the substrate for PanB, and produce competitive substrates for PanC; lowering PanB activity would be expected to increase α-KIV availability for α-HIV synthesis and correspondingly decrease the amount of pantoate synthesized. When α-KIV is added to the medium, all three strains produced significantly more HMBPA. This result implies that α-KIV is a precursor to HMBPA, as described in Figure 2, and that excess α-KIV favors HMBPA production. This result also suggests that synthesis of HMBPA is at least partially due to an overflow effect of excess α-KIV production. When pantoate was added to the medium, HMBPA was reduced by roughly 50 percent in all three strains. Conversely, the strains each produced significantly more pantothenate. This result is also consistent with the model that the two pathways produce competing substrates for PanC (α-HIV and pantoate). Taken together, the above results further indicate that increasing pantoate synthesis should be beneficial in promoting pantothenate production as well as reducing HMBPA levels. Moreover, factors that decrease pantoate synthesis negatively affect pantothenate synthesis.

Example IV. Effect of Increasing PanB and/or Regulating PanE1 on Production of Pantothenate

PA668 is a derivative of PA824 that contains extra copies of *P26 panB* amplified at the *vpr* or *panB* locus. PA668 was constructed using the *panB* expression vector (pAN636, SEQ ID NO:22) which allows for selection of multiple copies using chloramphenicol (Figure 9). The pAN636 *NotI* restriction fragment, missing the *E. coli* vector sequences, was ligated and then used to transform PA824 with selection on plates containing 5 μg/ml chloramphenicol. Transformants resistant to 30 μg/ml chloramphenicol were isolated and screened for pantothenate production in 48 hour test tube cultures. The isolates shown produce less HMBPA that PA824 (conversely producing about 10 percent more pantothenate than PA824). A second strain, called PA669, was constructed which is PA824 with extra copies of *P26 panE1* amplified at the *vpr* or *panE1* locus. Strain PA669 was constructed by transforming PA824 with the self-ligated *Not*I fragment of plasmid pAN637, SEQ ID NO:23 (Figure 10) with selection for resistance to chloramphenicol. Two isolates of PA669 were chosen for

further study; PA669-5 produces less PanE1 than PA669-7 as judged by SDS-PAGE analysis of total cell extracts made from the two strains.

Test tube cultures of strains PA824, PA668-2, PA668-24, and the two isolates of PA669 (PA669-5 and PA669-7) were grown in three different media (SVY, SVY + aspartate, and SVY + aspartate + pantoate) for 48 hours and then assayed for pantothenate, HMBPA, and β-alanine (Table 3).

Table 3. Effect of extra copies of panB and panE1 on pantothenate and HMBPA production by PA824, 48 hour test tube culture data, SVY medium.

			no additions			+ aspartate (10 g/L)			+ aspartate (10 g/L) & pantoate (5 g/L)		
Strain	***panB* plasmid**	***panE* plasmid**	**[pan] (g/L)**	**[β-ala] (g/L)**	**HMBPA ***	**[pan] (g/L)**	**[β-ala] (g/L)**	**HMBPA**	**[pan] (g/L)**	**[β-ala] (g/L)**	**HMBPA**
PA824	NONE	NONE	1.8	0.05	<0.1	4.7	2.5	0.53	5.6	2.5	<0.10
PA668-2	pAN636	NONE	1.5	<0.04	<0.1	5.0	1.6	<0.10	4.9	1.2	<0.10
PA668-24	pAN636	NONE	1.8	0.05	<0.1	4.9	2.8	0.34	6.1	2.6	<0.10
PA669-5	NONE	pAN637	1.8	0.04	<0.1	4.2	3.1	0.74	5.8	2.6	0.30
PA669-7	NONE	pAN637	1.8	0.06	<0.1	3.7	3.2	1.41	5.2	2.5	0.75

* HMBPA = peak area X 10^{-3}

None of the strains produced detectable quantities of HMBPA in SVY medium. All strains produced roughly equivalent amounts of pantothenate and low amounts of β-alanine indicating that β-alanine is limiting for both pantothenate and HMBPA synthesis in these cultures and that β-alanine is a precursor for both compounds. When grown in SVY + aspartate medium, the two PA669 isolates produced more HMBPA than PA824 whereas both PA668 isolates produced less HMBPA than PA824. It is noteworthy that the strain that produces the most PanE1 (PA669-7) produced the most HMBPA (and the least pantothenate). This suggests that high levels of PanE1 favor the production of HMBPA at the expense of lower pantothenate synthesis. It is also interesting that PA668-24 produced more HMBPA than PA668-2, even though SDS-PAGE analysis of extracts from the two strains showed that they produce roughly equivalent levels of PanB. The SDS-PAGE analysis also showed that PA668-24 makes much more IlvC than PA668-2. Based on these data, it is proposed that IlvC influences HMBPA synthesis by increasing steady state levels of α-KIV and/or by catalyzing α-HIV formation from α-KIV, thereby accounting for the observed shift towards production of HMBPA.

The final set of data in Table 3 shows that adding pantoate to the growth medium decreased HMBPA production by all strains that had previously produced detectable levels, *e.g.*, by shifting synthesis towards pantothenate. This further supports the model that α-HIV and pantoate are competitive substrates for PanC.

Example V: Increasing Pantothenate Synthesis by Reduction of Pantothenate Kinase in Production Strains

One strategy to increase pantothenate production is to reduce the amount of pantothenate kinase activity in production strains. Pantothenate kinase is the first enzyme in the pathway from pantothenate to Coenzyme A. It was hypothesized that lower pantothenate kinase activity would lead to lower steady state levels of Coenzyme A, which could in turn lead to higher PanB enzyme activity and higher titers of pantothenate. As described in U.S. patent application 09/667,569, two unlinked genes have been identified in *B. subtilis* that both encode pantothenate kinase, 1) *coaA*, which is homologous to the essential *E. coli* pantothenate kinase gene, and 2) *coaX*, which represents a novel class of bacterial pantothenate kinase genes.

Either *coaA* or *coaX* can be deleted from a wild type *B. subtilis* strain without any apparent effect on growth on rich or minimal medium. However, it is not possible to generate strains having a deletion in both genes, suggesting that one or the other is necessary for viability. Therefore, a strain was constructed having a deleted *coaX* and then *coaA* was mutated to reduce the specific activity of the CoaA enzyme. Although the phenotypes of the *ΔcoaX*, mutated *coaA* strains are subtle, the data is consistent with the hypothesis that PanB activity can be increased by restricting pantothenate kinase activity. This in turn decreases HMBPA production and increases pantothenate production.

Installation of* ΔcoaX *and mutated* coaA *alleles into PA824.

Deletion of *coaX* from PA824 was accomplished in a single step by transforming PA824 to kanamycin resistance with chromosomal DNA from PA876 (PY79 *ΔcoaX::kan*), described in U.S. patent application 09/667,569, to give PA880. The *caaX* deletion in PA880 and PA876 was derived ultimately from plasmid pAN336 (SEQ ID NO:26, see U.S. Patent Application Serial No. 09/667,569). Next, a control version of a wild type *coaA* gene and two mutated alleles of *coaA* were introduced as follows. The control and two mutated alleles were first introduced into the chromosome of wild type strain PY79 using transformation to chloramphenicol resistance by plasmids, and then chromosomal DNA from these intermediate strains was used to transform PA880 to chloramphenicol resistance. The plasmids used to integrate the control and mutant alleles were pAN294 (SEQ ID NO:27, see U.S. Patent Application Serial No. 09/667,569), pAN343, and pAN344. pAN343 is almost identical to pAN294, except that it contains a T to C base change at base number 3228 of pAN294. Similarly, pAN344 has a CC to TA change at base numbers 3217 and 3218 of pAN294. All three plasmids have a chloramphenicol resistance gene (*cat*) substituting for the dispensable gene of unkown funtion, *yqjT*, that lies just downstrean from *coaA*. The intermediate strains derived from PY79 by double crossovers of pAN294 (wild type *coaA, yqjT::cat*), pAN343 (*coaA2 Y155H, yqjT::cat*), and pAN344 (*coaA2 S151L, yqjT::cat*), are named PA886, PA887, and PA888, repectively. Next, PA880 was transformed to chloramphenicol resistance with chromosomal DNA from PA886, PA887, or PA888, to give strains PA892 (wild type *coaA, cat*), PA893 (*coaA* Y155H, *cat*), and PA894 (*coaA* S151L, *cat*), respectively. Eight candidates for PA893 were checked for acquisition of the Y155H mutation by PCR of the *coaA* gene and *Nla*III digestion, and all eight were correct.

Several candidates for each new strain, including PA880, were assayed for pantothenate production at 43° in standard test tube cultures grown in SVY medium plus 5 g/l β-alanine (see Table 4).

Table 4. Production of pantothenate and fantothenate by derivatives of PA824 that have been deleted for* coaX *and mutated for* coaA, *grown at 43°C in 48 hour test tube cultures of SVY glucose + β-alanine.

Strain	*coaA*	*coaX*	OD_{600}	[pan] g/l	[HMBPA] g/l
PA824	wt	wt	11.1	4.4	0.77
PA824	wt	wt	11.4	4.1	0.70
PA880	wt	Δ	11.6	5.5	0.33
PA880	wt	Δ	12.7	5.1	0.33
PA892-1	wt, *cat*	Δ	11.7	4.5	0.26
PA892-2	wt, *cat*	Δ	13.4	4.5	0.26
PA892-3	wt, *cat*	Δ	12.9	4.8	0.25
PA893-1	Y155H, *cat*	Δ	10.7	4.3	0.24
PA893-2	Y155H, *cat*	Δ	12.0	4.5	0.25
PA893-3	Y155H, *cat*	Δ	11.9	4.7	0.23
PA893-4	Y155H, *cat*	Δ	12.8	4.3	0.20
PA893-5	Y155H, *cat*	Δ	11.6	4.7	0.28
PA893-8	Y155H, *cat*	Δ	10.0	4.7	0.25
PA894-1	S151L ?, *cat*	Δ	11.6	4.6	0.29
PA894-2	S151L ?, *cat*	Δ	15.6	4.5	0.27
PA894-3	S151L ?, *cat*	Δ	12.3	5.0	0.31
PA894-4	S151L ?, *cat*	Δ	12.2	5.0	0.27
PA894-5	S151L ?, *cat*	Δ	11.8	4.5	0.26
PA894-6	S151L ?, *cat*	Δ	11.2	4.7	0.27
PA894-7	S151L ?, *cat*	Δ	13.1	4.7	0.26
PA894-8	S151L ?, *cat*	Δ	13.2	4.8	0.32

In medium containing β-alanine, PA894 (S151L), but not PA893 (Y155H) gave, on average, slightly higher pantothenate levels than PA892 (the isogenic strain with wild type *coaA*). PA880 gave significantly higher pantothenate (5.5 and 5.1 g/l) than its isogenic parent, PA824 (4.4 and 4.1 g/l), and slightly higher pantothenate levels than PA894 (average about 4.7 g/l). It is possible that the *ΔyqjT::cat* insertion present in PA892, PA893, and PA894 (but not PA880) has an effect that counteracts any gain that might result from the mutated *coaA* alleles.

Despite the narrow range of pantothenate titers from the new strains, a highly significant pattern was observed for production of HMBPA. In all strains where *coaX* was deleted, the HMBPA titer was two- to three-fold lower than for PA824. This is consistent with the principle that HMBPA production results in part from a limitation in PanB activity. Consequently, *ΔcoaX* leads to increased *PanB* activity.

Example VI: Increasing Pantothenate Synthesis by Combined Increase in PanB and Reduction of Pantothenate Kinase in Production Strains

PA668, which contains an extra PanB expression cassette that is designed to be amplified by chloramphenicol (see Example IV), produces more pantothenate and less HMBPA than its predecessor, PA824. Since deletion of *coaX* from PA824 also reduces HMBPA production and moderately improves pantothenate production (PA880), a strain combining the two modifications was constructed and tested for pantothenate production. Plasmid pAN636 (Figure 9) was digested with *NotI*, ligated into circles, and used to transform PA880 to chloramphenicol resistance to give strain PA911. Several isolates of PA911 were tested by PCR to determine whether the plasmid had integrated at *vpr* as intended, or at *panB*, where it can also integrate. Both types were found. One isolate of each type of PA911, PA911-5 and PA911-8, were amplified on tetracycline (*panD* cassette) and chloramphenicol (*panB* cassette) and tested for pantothenate production in test tube cultures grown in SVY plus β-alanine (see Table 1). Both isolates produced more pantothenate, and slightly less HMBPA than their parent, PA880. Moreover, consistent with the PA668 isolates, PA911-8, which has the *panB* cassette integrated at *panB*, produced the highest level of pantothenate.

Example VII: Increasing Pantothenate Production by Increasing Serine Availability

It was hypothesized that the ratio of pantothenate to HMBPA production could also be controlled by regulating the availability of serine in the microorganism cultures. In particular, it was proposed that increasing the availability of serine could increase pantothenate production relative to HMBPA production, whereas decreasing the availability of serine would decrease the production of pantothenate relative to HMBPA production. This method is based on the understanding that the PanB substrate, methylenetetrahydrofolate, is derived from serine. Thus, regulating serine levels should effectively regulate PanB substrate levels. To test this hypothesis, PA824 was grown in test tube cultures of SVY glucose plus 5 g/L β-alanine and ± 5 g/L serine for 48 hours and 43°C.

Table 5: ***Production of pantothenate and HMBPA by PA824 with and without the addition of serine***

serine added at 5 g/L	OD_{600}	[pan] g/L	[HMBPA] g/L
-	16.3	4.9	0.84
-	14.0	4.5	0.80
+	13.1	6.4	0.56
+	12.9	6.0	0.62

As demonstrated by the data presented in Table 5, addition of serine increases the level of production of pantothenate while conversely decreasing HMBPA production. As an alternative to feeding serine, another method of increasing serine and methylenetetrahydrofolate levels in order to regulate pantothenate production levels is to increase synthesis or the activity of 3-phosphoglycerate dehydrogenase or of serine hydroxymethyl transferase (the *serA* and *glyA* gene products, respectively), thereby increasing serine and methylenetetrahydrofolate biosynthesis in appropriately engineered microorganisms.

Example VIII: Pantothenate Production with strains PA668-2A and PA668-24 in 10-Liter Fermentors with Soy Flour Based Medium

Stains PA668-2A and PA668-24 were each grown twice in 10 liter fermentors. The medium was PFM-155 and the composition is as follows.

BATCH

	MATERIAL	g/L (final)
1	Amberex 1003	5
2	Cargill 200/20 (soy flour)	40
3	Na Glutamate	5
4	$(NH_4)_2SO_4$	8
5	$MgSO_4 \cdot 7H_2O$	1
6	MAZU DF204C	1
7	H_2O	qs to 4 L

Added After Sterilization and Cool Down

1	KH_2PO_4	10
2	$K_2HPO_4 \cdot 3H_2O$	20
3	H_2O	qs to 400 ml
1	80% Glucose	20
2	$CaCl_2 \cdot 2H_2O$	0.1
1	Sodium Citrate	1
2	$FeSO_4 \cdot 7H_2O$	0.01
3	SM-1000X	1 X

FEED

	MATERIAL	g/L (final)
1	80% Glucose	800
2	$CaCl_2 \cdot 2H_2O$	0.8
3	H_2O	qs to 3500 ml

Added After Sterilization and Cool Down

1	Sodium Citrate	2.0
2	$FeSO_4 \cdot 7H_2O$	0.02
3	SM-1000X	2 X
4	Glutamate Na	5.0
5	H_2O	qs to 500 ml

The pantothenate production by PA668-2A was 45 g/L and 51 g/L at 36 hours, similar to routine PA824 fermentations in the same medium. After 36 hours, when pantothenate production routinely begins to slow with PA824, both PA668-2A fermentations continued production to yield 63 g/l pantothenate at 48 hours. Most significantly, the production of HMPBA at 48 hours was reduced to 3-5 g/L, and was less than 5% of the pantothenate during most of the earlier fermentation. Clear benefits to pantothenate synthesis are evident from the increased levels of PanB in strain PA668. Strain PA668-24 produced pantothenate at an even faster rate with the two fermentations averaging 58 g/L after 36 hours.

Equivalents Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed:

1. A process for the production of a HMBPA-free pantothenate composition, comprising

culturing a microorganism having a deregulated pantothenate biosynthetic pathway under conditions such that a HMBPA-free pantothenate composition is produced.

2. The process of claim 1, wherein said microorganism has at least two pantothenate biosynthetic enzymes deregulated.

3. The process of claim 1, wherein said microorganism has at least three pantothenate biosynthetic enzymes deregulated.

4. The process of claim 1, wherein said microorganism has at least four pantothenate biosynthetic enzymes deregulated.

5. The process of claim 4, wherein said microorganism has a deregulated ketopantoate hydroxymethyltransferase, a deregualted ketopantoate reductase, a deregulated pantothenate synthetase and a deregulated aspartate-α-decarboxylase.

6. The process of any one of claims 1 to 5, wherein said microorganism further has a deregulated isoleucine-valine (*ilv*) biosynthetic pathway.

7. The process of claim 6, wherein said microorganism has at least two isoleucine-valine (*ilv*) biosynthetic enzymes deregulated.

8. The process of claim 6, wherein said microorganism has at least three isoleucine-valine (*ilv*) biosynthetic enzymes deregulated.

9. The process of claim 8, wherein said microorganism has a deregulated acetohydroxyacid acid synthetase, a deregulated acetohydroxyacid isomeroreductase, and a deregulated dihydroxyacid dehydratase.

10. A process for the production of a HMBPA-free pantothenate composition comprising culturing a microorganism having a deregualted pantothenate biosynthetic pathway and a deregulated isoleucine-valine (*ilv*) biosynthetic pathway, said microorganism having PanB activity regulated such that a HMBPA-free pantothenate composition is produced.

11. A process for the production of a HMBPA-free pantothenate composition comprising culturing a microorganism having a deregualted pantothenate biosynthetic pathway and a deregulated isoleucine-valine (*ilv*) biosynthetic pathway, said microorganism having PanE1 activity regulated such that a HMBPA-free pantothenate composition is produced.

12. A process for the production of a HMBPA-free pantothenate composition comprising culturing a microorganism having a deregualted pantothenate biosynthetic pathway and a deregulated isoleucine-valine (*ilv*) biosynthetic pathway, said microorganism having PanE2 activity regulated such that a HMBPA-free pantothenate composition is produced.

13. A process for the production of a HMBPA-free pantothenate composition comprising culturing a microorganism having a deregualted pantothenate biosynthetic pathway and a deregulated isoleucine-valine (*ilv*) biosynthetic pathway, said microorganism having IlvC activity regulated such that a HMBPA-free pantothenate composition is produced.

14. A process for the production of a HMBPA-free pantothenate composition comprising culturing a microorganism having a deregualted pantothenate biosynthetic pathway and a deregulated isoleucine-valine (*ilv*) biosynthetic pathway, said microorganism having PanB and PanE1 activites regulated such that a HMBPA-free pantothenate composition is produced.

15. A process for the production of a HMBPA-free pantothenate composition comprising culturing a microorganism having a deregualted pantothenate biosynthetic pathway and a deregulated isoleucine-valine (*ilv*) biosynthetic pathway, said microorganism having PanB and PanE2 activites regulated such that a HMBPA-free pantothenate composition is produced.

16. A process for the production of a HMBPA-free pantothenate composition comprising culturing a microorganism having a deregualted pantothenate biosynthetic pathway and a deregulated isoleucine-valine (*ilv*) biosynthetic pathway, said microorganism having PanB and IlvC activites regulated such that a HMBPA-free pantothenate composition is produced.

17. The process of any one of claims 10, 14, 15 and 16, wherein PanB activity is increased by overexpressing PanB.

18. The process of any one of claims 10, 14, 15 and 16, wherein PanB activity is increased by expressing multiple copies of the *panB* gene.

19. The process of any one of claims 10, 14, 15 and 16, wherein Pan B activity is increased by decreasing feedback inhibition of PanB.

20. The process of claim any one of claims 10, 14, 15 and 16, wherein PanB activity is increased by regualting pantothenate kinase activity.

21. The process of claim 20, wherein pantothenate kinase activity is decreased.

22. The process of claim 21, wherein CoaA is deleted and CoaX is downregulated.

23. The process of claim 21, wherein CoaX is deleted and CoaA is downregulated.

24. The process of claim 21, wherein CoaX and CoaA are downregulated.

25. The process of claim 10, 14 or 15, wherein PanE activity is decreased by deleting the *panE2* gene.

26. The process of claim 10, 14 or 15, wherein PanE activity is decreased by regulating expression of the *panE* gene.

27. The process of claim 13 or 15, wherein IlvC activity is decreased by regulating expression of the *ilvC* gene.

28. The process of any one of the above claims, wherein said microorganism is cultured under conditions of reduced steady state glucose.

29. The process of any one of the above claims, wherein said microorganism is cultured under conditions of increased steady state dissolved oxygen.

30. The process of any one of the above claims, wherein said microorganism is cultured under conditions of excess serine.

31. The process of any one of the above claims, wherein said microorganism has the pantothenate biosynthetic pathway deregualted such that pantothenate production is independent of β-alanine feed.

32. The process of claim any one of the above claims, wherein said microorganism overexpresses the *panD* gene such that pantothenate production is independent of β-alanine feed.

33. The process of any one of the above claims wherein the microorganism belongs to the genus *Bacillus*.

34. The process of any one of the above claims, wherein the microorganism is *Bacillus subtilis*.

35. A product synthesized according to the process of any one of the above claims.

36. A HMBPA-free pantothenate composition synthesized according to the process of any one of the above claims.

37. A composition synthesized by a microorganism having a deregulated pantothenate biosynthetic pathway, wherein said composition comprises pantothenate and is essentially free of HMBPA.

38. A recombinant microorganism for the production of HMBPA-free compositions of pantothenate, said microorganism having a deregulated pantothenate biosynthetic pathway, a deregulated isoleucine-valine (*ilv*) pathway, and having PanB or PanE1 selectively regulated.

39. A recombinant microorganism for the production of HMBPA-free compositions of pantothenate, said microorganism having a deregulated pantothenate biosynthetic pathway, a deregulated isoleucine-valine (*ilv*) pathway, and having PanB and PanE1 selectively regulated.

40. A recombinant microorganism for the production of HMBPA-free compositions of pantothenate, said microorganism having a deregulated pantothenate biosynthetic pathway, a deregulated isoleucine-valine (*ilv*) pathway, and having PanB or PanE2 selectively regulated.

41. A recombinant microorganism for the production of HMBPA-free compositions of pantothenate, said microorganism having a deregulated pantothenate biosynthetic pathway, a deregulated isoleucine-valine (*ilv*) pathway, and having PanB and PanE2 selectively regulated.

42. A recombinant microorganism for the production of HMBPA-free compositions of pantothenate, said microorganism having a deregulated pantothenate biosynthetic pathway, a deregulated isoleucine-valine (*ilv*) pathway, and having PanE1 deleted, PanE2 deleted and IlvC overexpressed.

43. The microorganism of any one of claims 38-42 belonging to the genus *Bacillus*.

44. The microorganism of any one of claims 38-42 which is *Bacillus subtilis*.

45. A process for the production of a selectively mixed pantothenate:HMBPA composition, comprising

culturing a microorganism having a deregulated pantothenate biosynthetic pathway under conditions such that a selectively mixed pantothenate:HMBPA composition is produced.

46. The process of claim 45, wherein the microorganism is cultured under conditions that favor pantothenate production.

47. The process of claim 45, wherein the microorganism is cultured under conditions that favor HMBPA production.

48. The process of claim 45 or 46, wherein the microorganism is cultured under conditions of controlled steady state glucose that favor pantothenate production.

49. The process of claim 45 or 47, wherein the microorganism is cultured under conditions of controlled steady state glucose that favor HMBPA production.

50. The process of claim 45 or 46, wherein the microorganism is cultured under conditions of controlled steady state dissolved oxygen that favor pantothenate production.

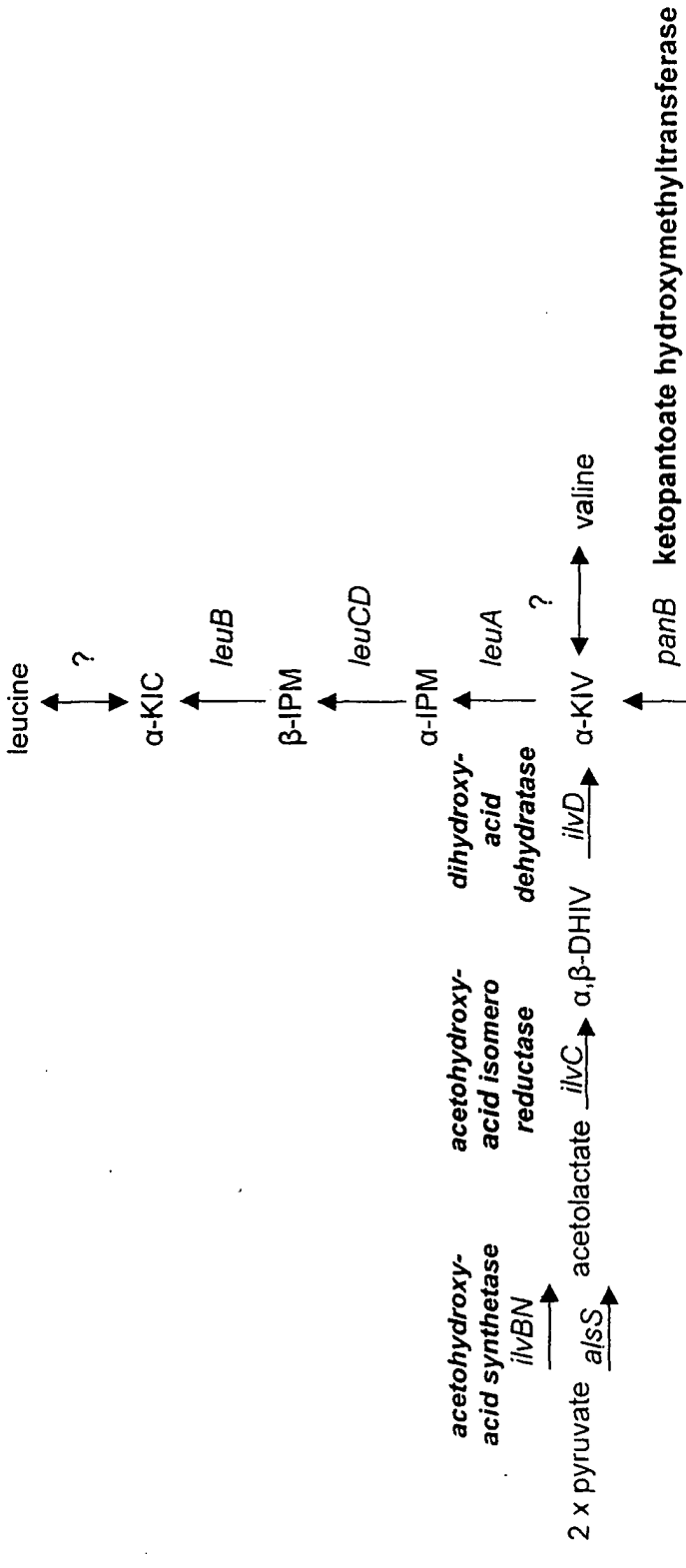
51. The process of claim 45 or 47, wherein the microorganism is cultured under conditions of controlled steady state dissolved oxygen that favor HMBPA production.

52. The process of claim 45 or 46, wherein the microorganism is cultured under conditions of controlled serine levels that favor pantothenate production.

53. The process of claim 45 or 46, wherein the microorganism has modifications that favor pantothenate production.

54. The process of claim 45 or 47, wherein the microorganism has modifications that favor HMBPA production.

55. A product produced by the process of any one of claims 45-54.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ketopantoate
panE1 ketopantoate reductase
pantoate
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acetohydroxy-acid synthetase ilvBN
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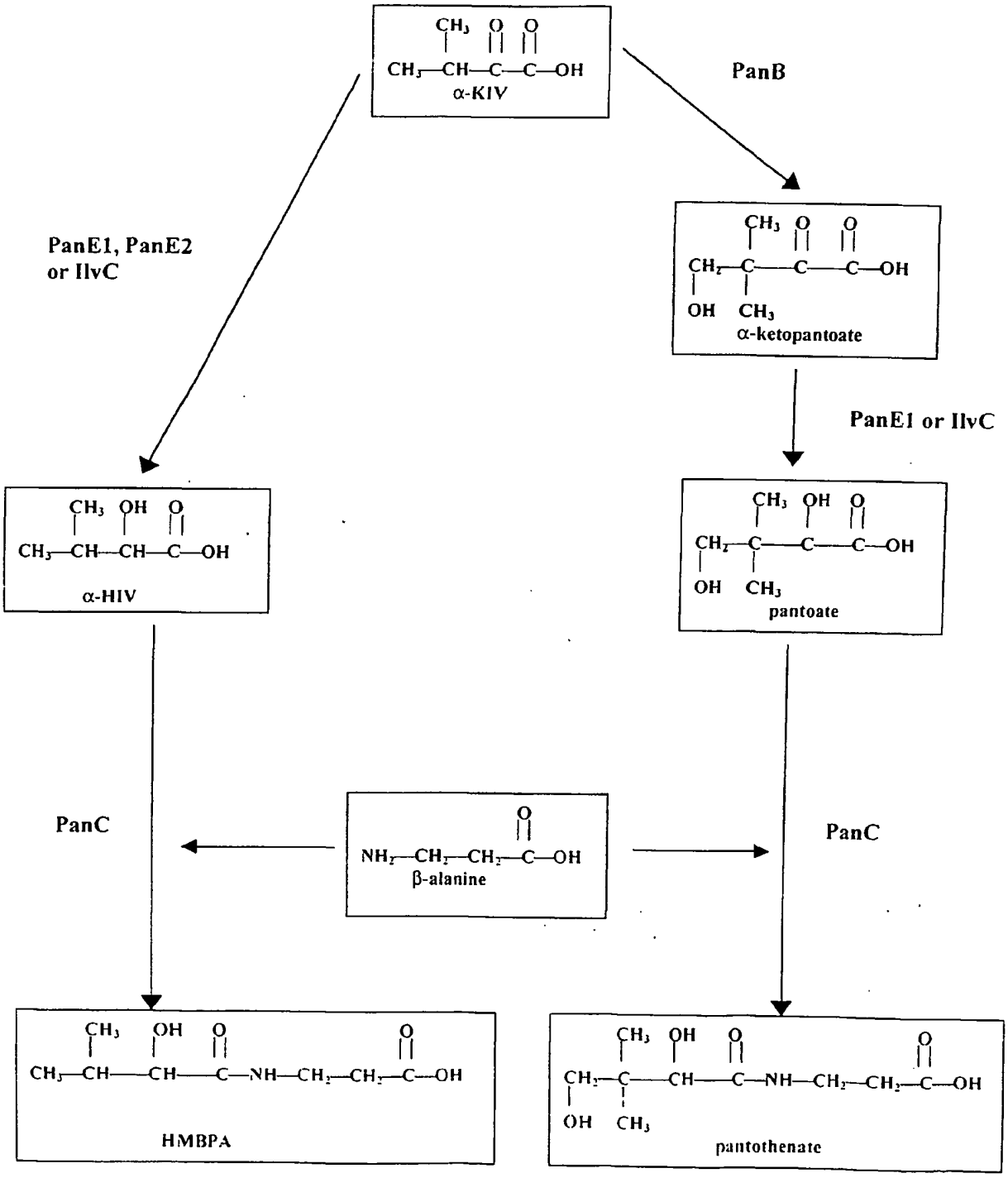
FIG 1

Figure2. Proposed pathway for biosynthesis of HMBPA.



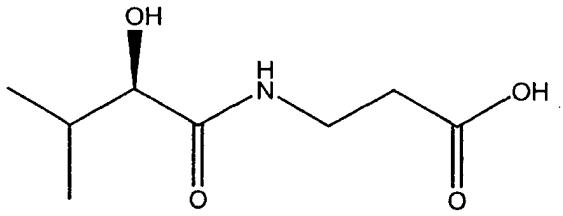


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FIG 3

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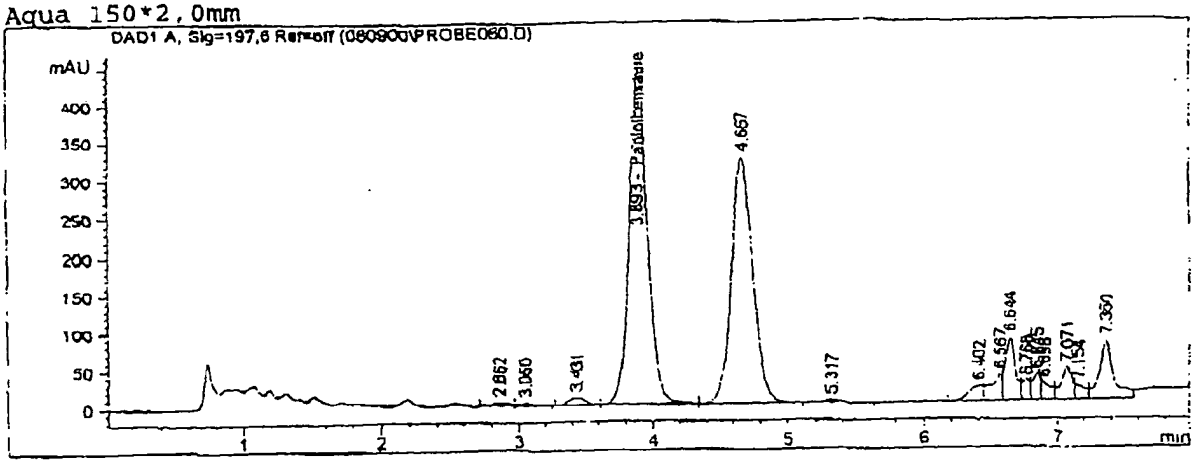

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Totals :                                  407.67936

 Results obtained with enhanced integrator!
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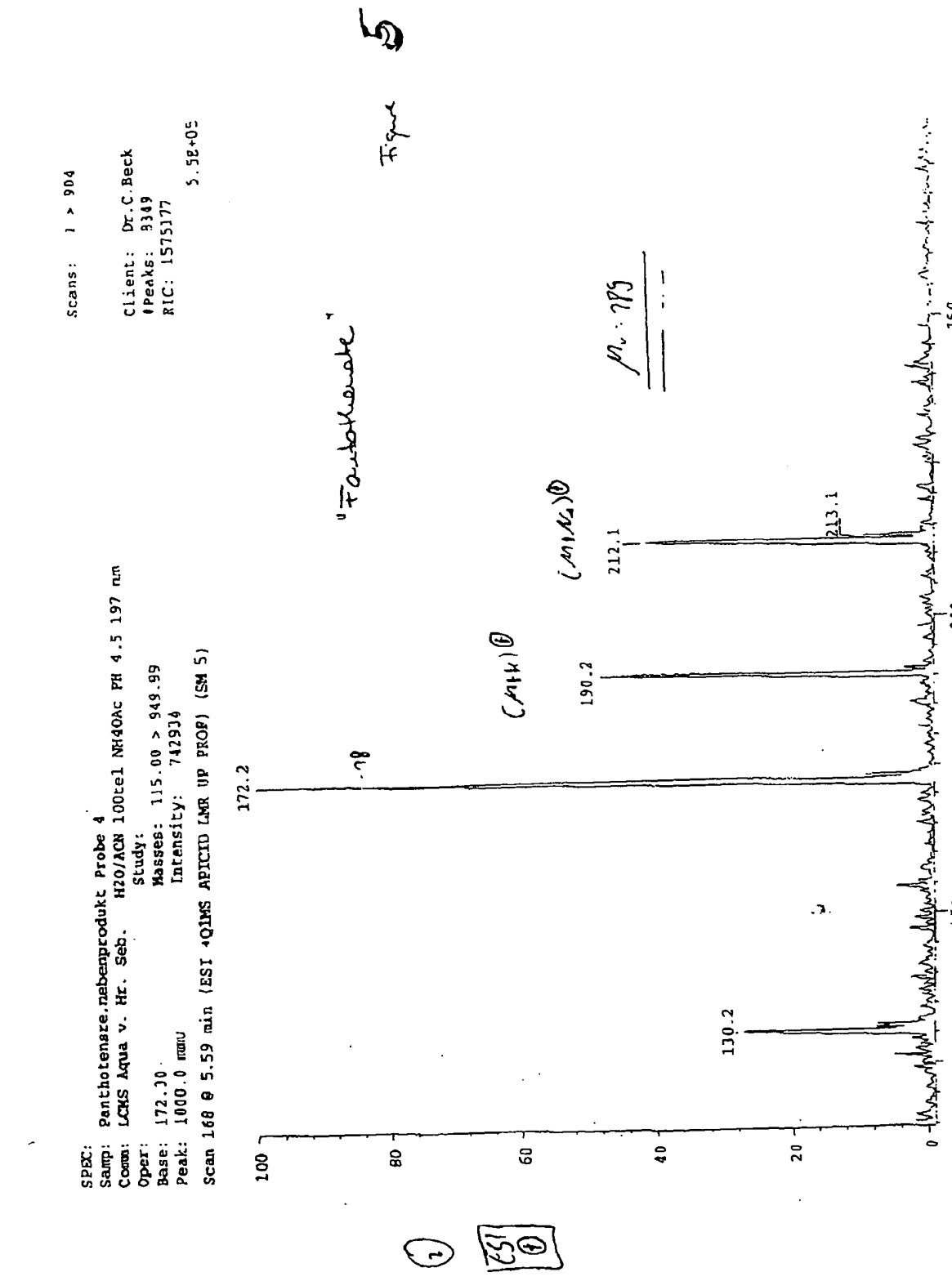
*** End of Report ***

HPLC - analysis of fermentation broth

Figure 4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Peak: 1000.0 mmmU
Study:
Masses: 115.00 > 949.99
Intensity: 742934
Client: Dr.C.Beck
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5.5E+05
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190.2
212.1
213.1
100
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60
40
20
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150
200
"Panthotenate"
Figure 5
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(1)
ESI ⊕

Figure 6. An alignment of the C-terminal amino acids from all known or suspected PanB proteins.

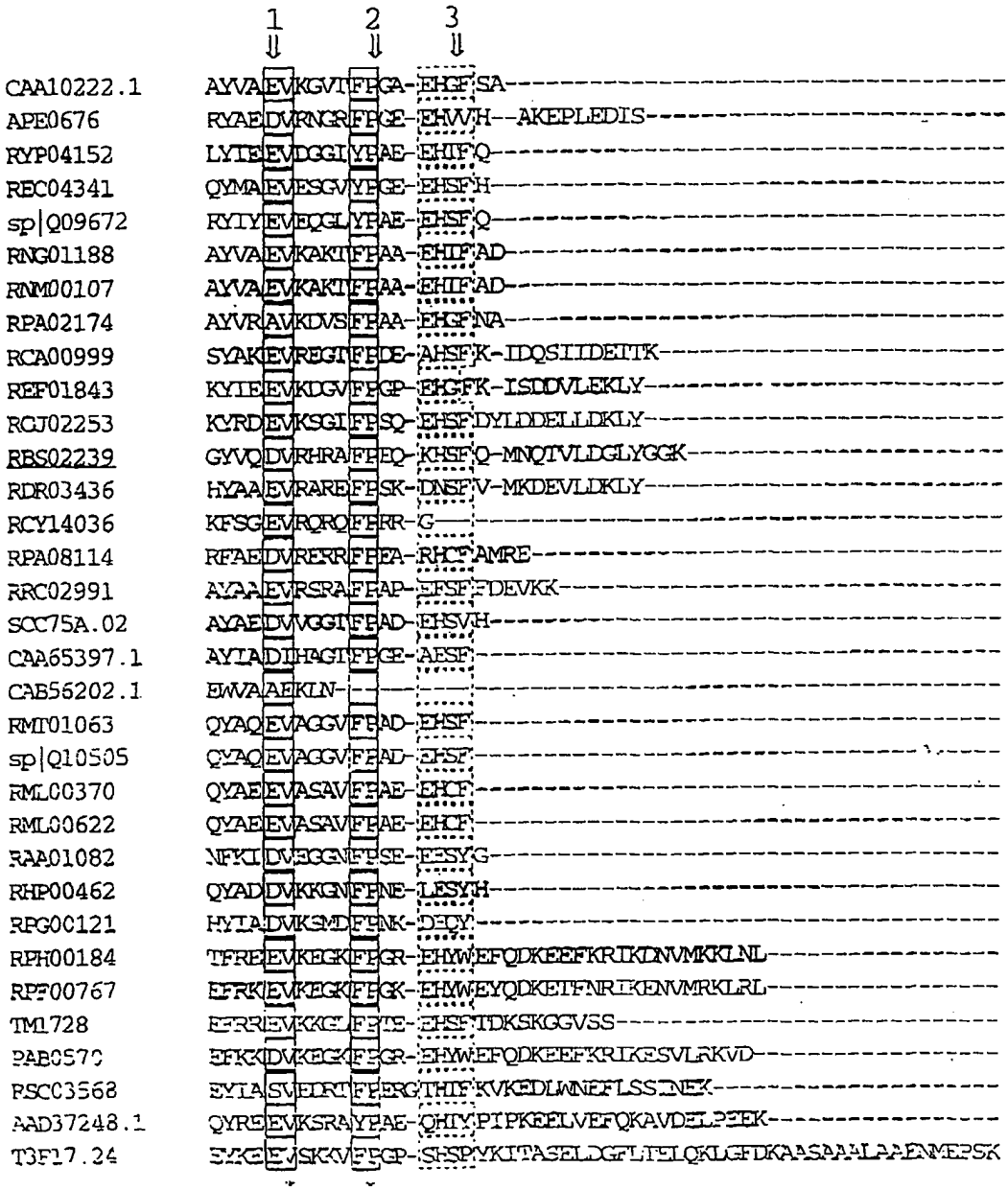


Figure 7. Construction of pAN624.

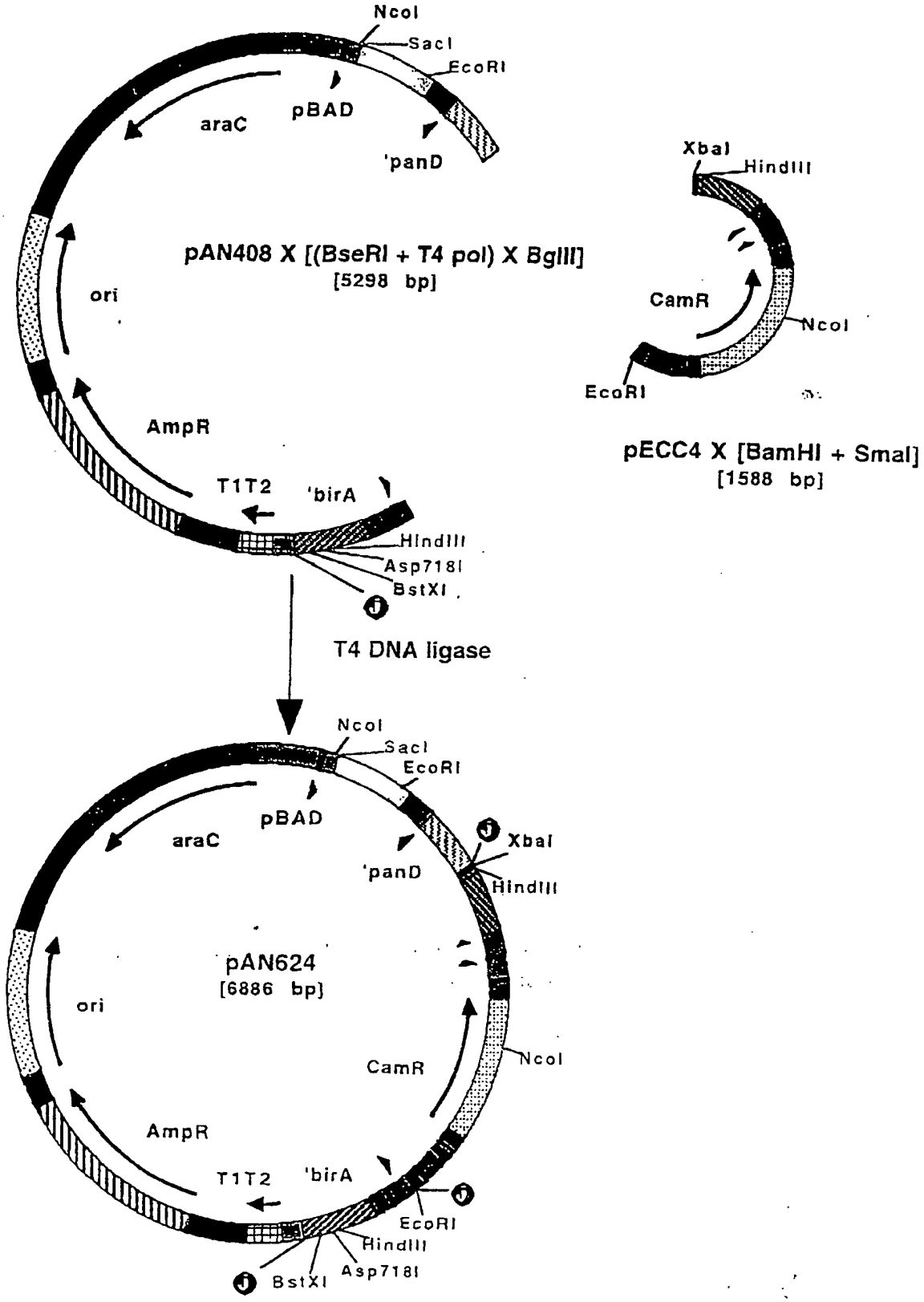


Figure 8. Construction of pAN620.

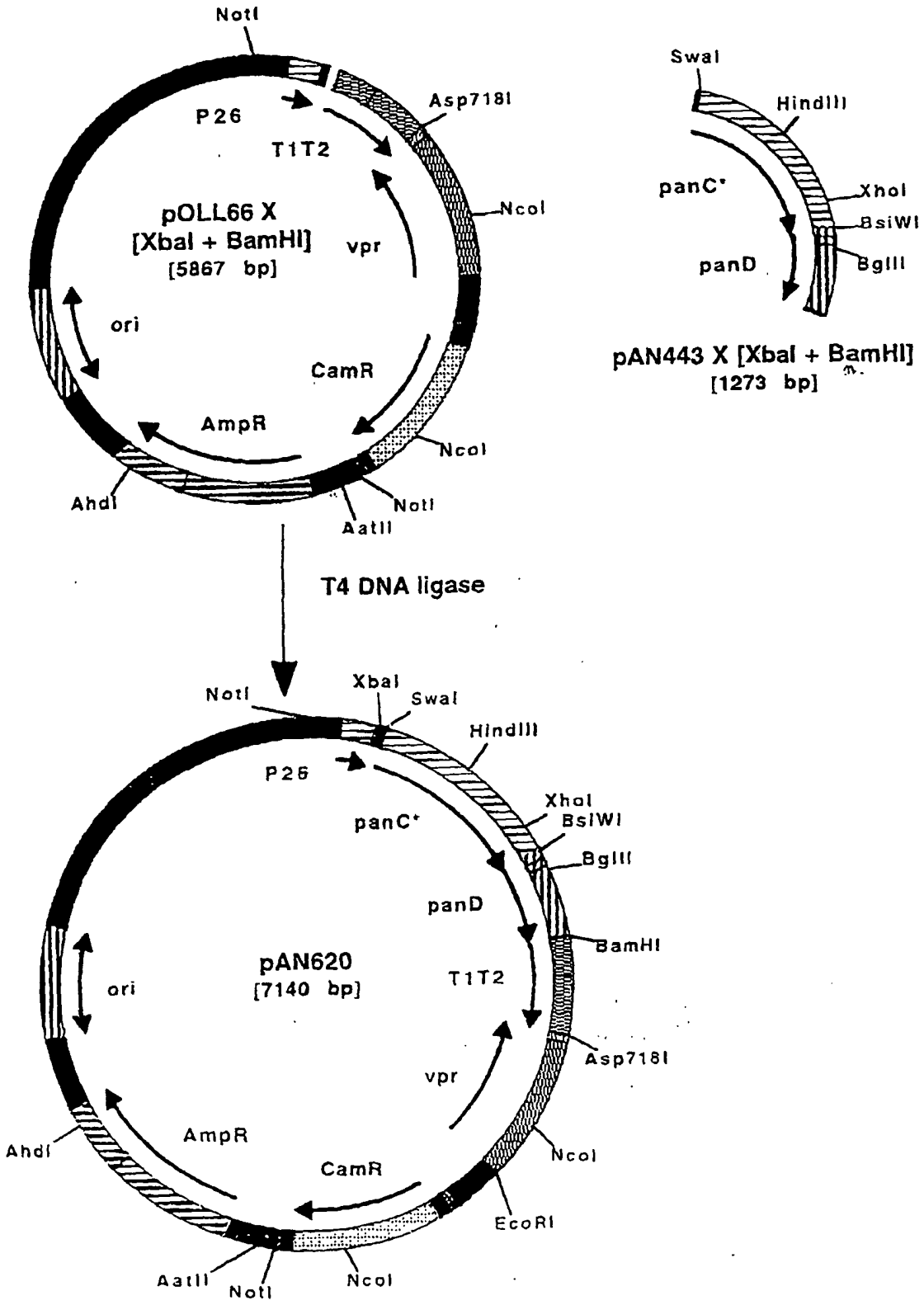


Figure 9 . Construction of pAN636.

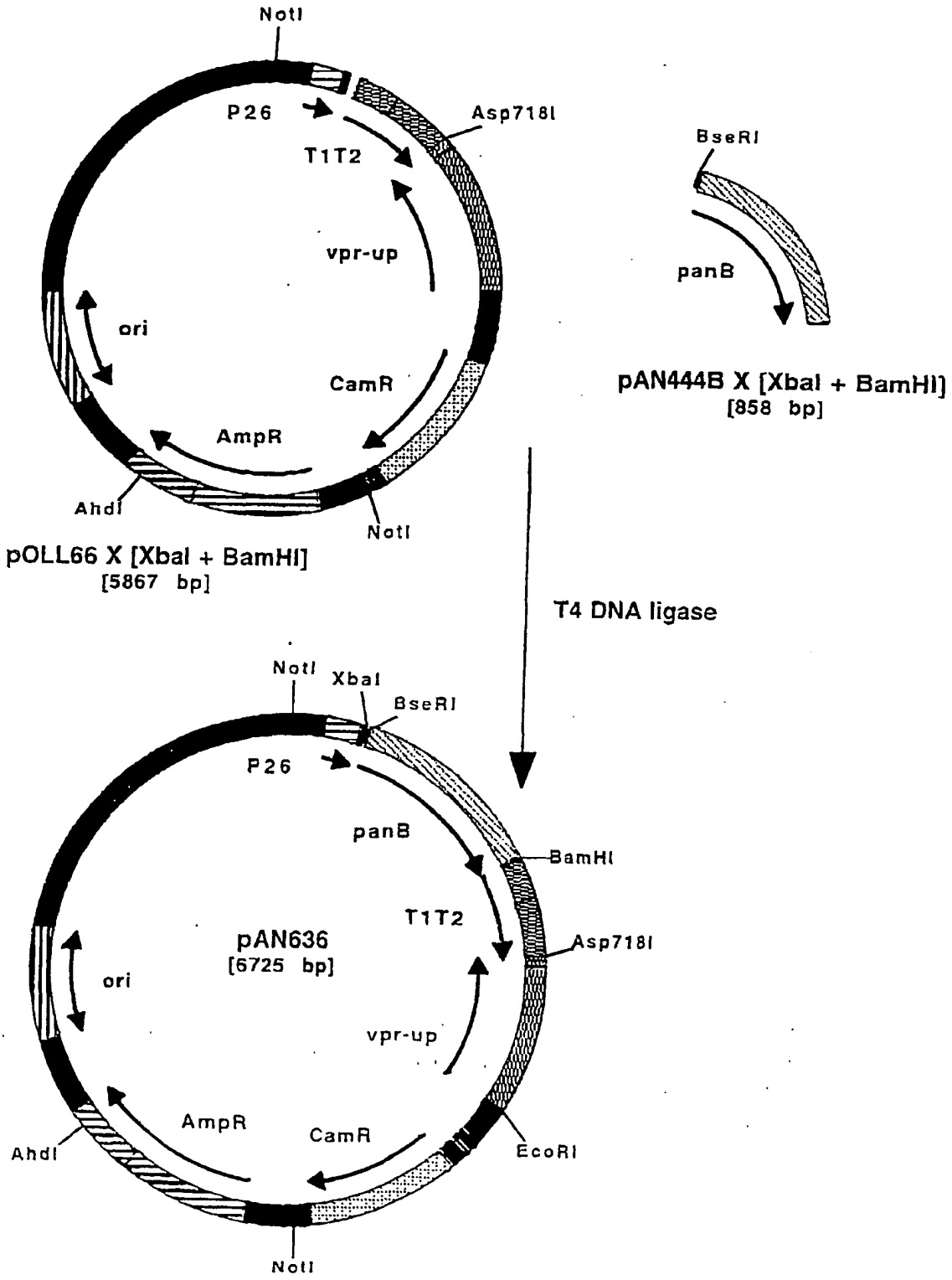
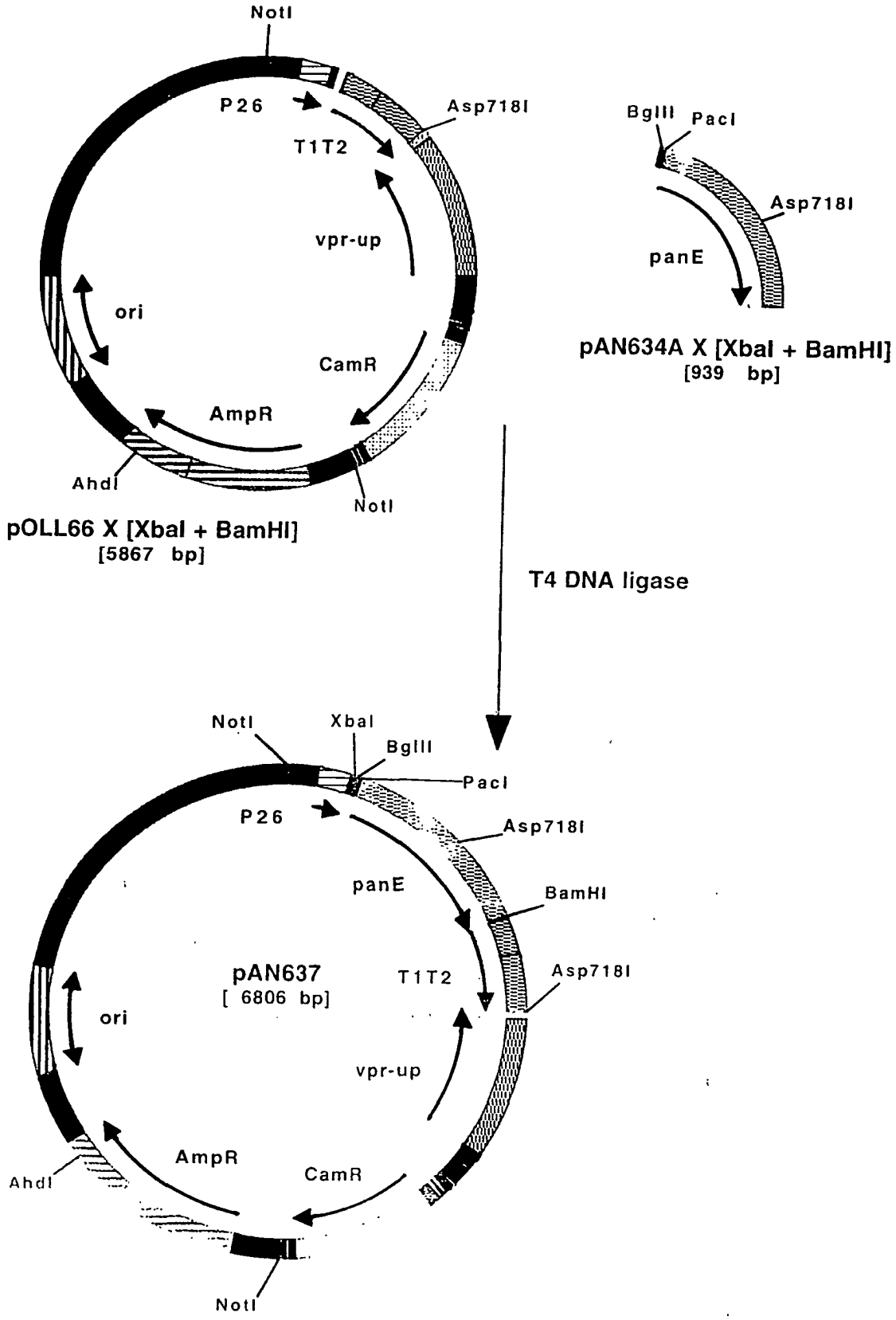


Figure 10 . Construction of pAN637.



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(54) Title: PROCESSES FOR ENHANCED PRODUCTION OF PANTOTHENATE

(57) Abstract: The present invention features improved methods for producing pantoate and pantothenate utilizing microorganisms having modified pantothenate biosynthetic enzyme activities. In particular, the invention features methods for reducing byproduct formation and increasing yields and purity of desired product. Recombinant microorganisms and conditions for culturing same are also are featured. Also featured are compositions produced by such microorganisms.

INTERNATIONAL SEARCH REPORT

PCT/US 02/01842

A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C12N

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EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search repon
10 April 2003	24/04/2003

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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